**Universidade Nova de Lisboa**
**Faculdade de Ciências Médicas**

# DNA DAMAGE INDUCED BY ACRYLAMIDE: ROLE OF GENETIC POLYMORPHISMS IN DNA DAMAGE LEVELS

**Marta Sofia Pereira Pingarilho Fazendeiro**

**Doutoramento em Ciências da Vida**

(Especialidade Genética)

**2013**

**Universidade Nova de Lisboa**
**Faculdade de Ciências Médicas**

# DNA DAMAGE INDUCED BY ACRYLAMIDE: ROLE OF GENETIC POLYMORPHISMS IN DNA DAMAGE LEVELS

**Marta Sofia Pereira Pingarilho Fazendeiro**

**Doutoramento em Ciências da Vida**

(Especialidade Genética)

**This thesis was supervised by:**

**Supervisor:** Professor Doutor Jorge Francisco Gaspar

**Co-supervisor:** Professor Doutor José Rueff

**2013**

**Acknowledgments**

I want to acknowledgment tightly to my supervisor Professor Jorge Gaspar, who invited and convinced me to join to this scientific journey. Thank you for giving me the opportunity to play a quality scientific work, for your mentorship, availability and scientific rigor and especially for your friendship and support.

I want to express my deepest gratitude to Professor José Rueff director of department of Genetics of Faculdade de Ciências Médicas da Universidade Nova de Lisboa for being my co-supervisor and for received me in this research group with so kindness and enormous friendship.

My deepest gratitude to Professor Nuno Oliveira for all comments, opinions and constant help those were so important throughout this work. Thank you above all for your friendship.

I want to express my acknowledgments to all colleagues and friends of the Genetic department of Faculdade de Ciências Médicas da Universidade Nova de Lisboa. Each one collaborated with important knowledge that made me grow up in scientific and also in personal terms. Everyone was very important, but I must emphasize the help and friendship of Professor Sebastião Rodrigues, Célia Martins, Bruno Gomes, Susana Silva and João Pereira de Lima. Thank you very much.

A very special thanks to my family, specially to my fathers, because they always believed it would be possible. Thank you for your constant support and encouragement. A special thanks to João Pedro and my daughters Carminho and Leonor that had to share my attention and time with this thesis

Finally, I want to acknowledge Fundação Calouste Gulbenkian that financially supported the work presented in this thesis, through the Grant 76438. I also thank you Fundação para a Ciência e Tecnologia that funded this work through the Project PTDC/SAU-OSM/105572/208 and through my PhD fellowship (SFRH/BD/22612/2005).

**Abstract**

Acrylamide (AA) has been classified as a probable human carcinogen by IARC. Besides being used in numerous industrial applications, AA is also present in a variety of starchy cooked foods. This AA exposure scenario raised concerns about risk in human health and suggests that the oral consumption of AA is an additional risk factor for cancer. A considerable number of findings strongly suggest that the reactive metabolite glycidamide (GA), an epoxide generated presumably by cytochrome P450 2E1, plays a central role in AA carcinogenesis.

Until now there are a scarcity of results concerning the mechanisms of genotoxicity of AA and GA in mammalian cells. In view of that, the study described in this thesis aims to unveil the genetic consequences of AA and GA exposure using mammalian cells as a model system.

With this aim we evaluated the cytotoxicity of AA and GA using the MTT assay and subsequently performed two cytogenetic end-points: chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs), in order to evaluate DNA damage induced by these compounds in V79 Chinese hamster cell line. The results showed that GA was more cytotoxic and clastogenic than AA.

Within the scope of this thesis the quantification of specific DNA adducts were also performed, namely N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua) and N3-(2-carbamoyl-2-hydroxyethyl)adenine (N3-GA-Ade). Interestingly, the GA concentration and the levels of N7-GA-Gua presented a linear dose-response relationship. Further, a very good correlation between the levels of N7-GA-Gua and the extent of SCEs were observed.

In order to understand the mechanisms of AA-induced toxicity, the modulation of reduced glutathione (GSH)-dependent mechanisms were studied, namely the evaluation of the effect of buthionine sulfoximine (BSO), an effective inhibitor of GSH synthesis, of GSH-monoethyl ester (GSH-EE), a cell permeable compound that is intracellularly hydrolysed to GSH and also of GSH endogenously added to culture medium,z in V79 cell line. The overall results reinforced the role of GSH in the modulation of the cytotoxic and clastogenic effects induced by AA.

Complementary to the studies performed in V79 cells, SCEs, specific DNA-adducts and alkaline comet assay in lymphocytes from healthy donors exposed to AA and GA were also evaluated. Both, the frequency of SCE and the quantification of specific GA DNA adducts, produced comparable results with those obtained in V79 cell line, reinforcing the idea that GA is far more genotoxic than AA. Further, the DNA damaging potential of AA and GA in whole blood leukocytes evaluated by the alkaline comet assay, showed that GA, but not AA, increases DNA damage.

Additionally, this study aimed to identify associations between DNA damage and biomarkers of susceptibility, concerning individual genetic polymorphisms involved in detoxification and DNA repair pathways (BER, NER, HRR and NHEJ) on the GA-induced genotoxicity assessed by the SCE assay and by the alkaline comet assay. The extent of DNA damage determined by the levels of SCEs induced by GA seems to be modulated by *GSTP1* (Ile105Val) and *GSTA2* (Glu210Ala) genotypes. Moreover, the results obtained from the comet assay suggested associations between DNA damage and polymorphisms of BER (*MUTYH* Gln335His and *XRCC1* Gln399Arg) and NER (*XPC* Ala499Val and Lys939Gln) genes, either alone or in combination.

The overall results from this study contribute to a better understanding of the genotoxicity and carcinogenicity of AA and GA in mammalian cells, as well as the knowledge about the variability in individual susceptibility involved in detoxification and repair of DNA damage due to these dietary xenobiotics.

**Keywords:** acrylamide; glycidamide; sister chromatid exchange; chromosomal aberrations; DNA-adducts; comet assay; SNPs; genotoxicity; carcinogenicity.

**Resumo**

Em 1994 a acrilamida (AA) foi classificada pela IARC como um provável cancerígeno para o homem. Para além da utilização de AA em numerosas aplicações industriais, a AA está também presente numa grande variedade de alimentos ricos em amido e processados a temperaturas elevadas. Esta exposição através da ingestão de produtos alimentares despoletou elevadas preocupações ao nível do risco para a saúde pública e poderá implicar um risco adicional para o aparecimento de cancro. A glicidamida (GA), o metabolito epóxido formado a partir da oxidação da AA provavelmente através do citocromo P450 2E1, é considerada por vários estudos, o principal responsável pela carcinogenicidade da AA.

Actualmente existe uma escassez de resultados relativamente aos mecanismos de genotoxicidade da AA e GA em células de mamífero. Por este motivo, o objectivo deste estudo centra-se na avaliação das consequências genéticas da exposição à AA e GA, recorrendo-se para tal ao uso de células de mamífero como modelo.

Tendo como base este objectivo avaliou-se a citotoxicidade da AA e GA, através do ensaio do MTT, e realizaram-se dois testes citogenéticos, o teste das aberrações cromossómicas (CAs) e o teste da troca de cromátides irmãs (SCEs), de modo a avaliar as lesões de DNA induzidas por estes compostos em células de hamster Chinês V79. Os resultados globalmente mostraram que a GA é mais citotóxica e clastogénica do que a AA.

No âmbito deste trabalho, foi também efectuada a quantificação de aductos específicos de DNA, nomeadamente N7-(2-carbamoil-2-hidroxietil)guanina (N7-GA-Gua) e N3-(2-carbamoil-2-hidroxietil)adenina (N3-GA-Ade). Os resultados obtidos permitem afirmar que os níveis de N7-GA-Gua e a concentração de GA apresentam uma relação linear dose-resposta. Foi também identificada uma óptima correlação entre os níveis de N7-GA-Gua e a frequência de troca de cromátides irmãs.

Adicionalmente, e de forma a compreender os mecanismos de toxicidade da AA, estudaram-se os mecanismos dependentes da modulação do glutationo reduzido (GSH), nomeadamente da butionina sulfoximina (BSO), um inibidor da síntese de GSH, do GSH-monoetil estér (GSH-EE), um composto permeável nas células e que é intra-celularmente hidrolisado a GSH e ainda do GSH adicionado exogenamente ao meio de

cultura, em células V79. Os resultados obtidos reforçaram o papel da modulação do GSH nos efeitos de citotoxicidade e clastogenicidade da AA.

Para além dos estudos efetuados com células V79, procedeu-se também à determinação da frequência de SCEs, à quantificação de aductos específicos de DNA, bem como ao ensaio do cometa alcalino em amostras de dadores saudáveis expostos à AA e GA. Tanto os resultados obtidos através do ensaio das SCE, como pela quantificação de aductos específicos de DNA, ambos efectuados em linfócitos estimulados, originaram resultados comparáveis aos obtidos anteriormente para as células V79, reforçando a ideia de que a GA é bastante mais genotóxica do que a AA. Por outro lado, os resultados obtidos pelo ensaio do cometa para exposição à AA e GA mostraram que apenas esta última aumenta o nível das lesões de DNA.

Outro objectivo deste trabalho, foi a identificação de possíveis associações existentes entre as lesões de DNA, quantificadas através do ensaio das SCEs e do cometa, e biomarcadores de susceptibilidade, tendo em conta os polimorfismos genéticos individuais envolvidos na destoxificação e nas vias de reparação do DNA (BER, NER, HRR e NHEJ) em linfócitos expostos à GA. Tal permitiu identificar associações entre os níveis de lesão de DNA determinados através do ensaio das SCEs, e os polimorfismos genéticos estudados, apontando para uma possível associação entre o *GSTP1* (Ile105Val) e *GSTA2* (Glu210Ala) e a frequência de SCEs. Por outro lado, os resultados obtidos através do ensaio do cometa sugerem uma associação entre as lesões de DNA e polimorfismos da via BER (*MUTYH* Gln335His e *XRCC1* Gln39Arg) e da via NER (*XPC* Ala499val e Lys939Gln), considerando os genes isoladamente ou combinados.

Estes estudos contribuem para um melhor entendimento da genotoxicidade e carcinogenicidade da AA e GA em células de mamífero, bem como da variabilidade da susceptibilidade individual na destoxificação e reparação de lesões de DNA provocadas pela exposição a estes xenobióticos alimentares.

**Palavras chave:** acrilamida; glicidamida; troca de cromátides irmãs; aberrações cromossómicas, aductos de DNA; ensaio do cometa; SNPs; genotoxicidade; carcinogenicidade.

**List of publications and communications**

From the results presented in this thesis, the following papers were published in international refereed journals:

**1.** Induction of Sister Chromatid Exchange by acrylamide and glycidamide in human lymphocytes: Role of polymorphisms in detoxification and DNA-repair genes in the genotoxicity of glycidamide
**Pingarilho M**, Oliveira NG, Martins C, Gomes BC, Fernandes AS, Martins V, Labilloy A, Lima JP, Rueff J and Gaspar JF.
Mutation Research (2013), 752, 1-7.

**2.** Genetic polymorphisms in detoxification and DNA repair genes and susceptibility to glycidamide-induced DNA damage
**Pingarilho M**, Oliveira NG, Martins C, Fernandes AS, Lima JP, Rueff J and Gaspar JF.
Journal of Toxicology and Environmental Health, Part A (2012) 75:13-15, 920-933.

**3.** Cytotoxicity and chromosomal aberrations induced by acrylamide in V79 cells: role of glutathione modulators.
Oliveira NG, **Pingarilho M**, Martins C, Fernandes AS, Vaz S, Martins V, Rueff J, Gaspar JF.
Mutation Research (2009) 676, 87-92.

**4.** Cytogenetic damage induced by acrylamide and glycidamide in mammalian cells: correlation with specific glycidamide-DNA adducts.
Martins C, Oliveira NG, **Pingarilho M**, Gamboa da Costa G, Martins V, Marques MM, Beland FA, Churchwell MI, Doerge DR, Rueff J, Gaspar JF.
Toxicology Science (2007) 95, 383-390.

This thesis also contains methods published in the following papers:

**1.** Genotoxic effects of occupational exposure to lead and influence of polymorphisms in genes involved in lead toxicokinetics and in DNA repair
García-Lestón J, Roma-Torres J, Vilares M, Pinto R, Prista J, Teixeira JP, Mayan O, Conde J, **Pingarilho M**, Gaspar JF, Pásaro E, Méndez J, Laffon B.
Environment International (2012), 43: 29-36.

**2.** Genotoxic effects of doxorubicin in cultured human lymphocytes with different glutathione S-transferase genotypes
Ramos DL. Gaspar JF, **Pingarilho M**, Gil OM, Fernandes AS, Rueff J, Oliveira NG
Mutation Research (2011), 724: 28-34.

The results related with this study were also presented in scientific meetings in the following poster communications:

**1.** Gene expression induced by Acrylamide and Glycidamide in mammalian cells.
**Pingarilho M**, Lima JP, Martins C, Rueff J, Gaspar JF
Post-GWAS Horizons in Molecular Epidemiology: Digging Deeper into the Environment, Genome Wide Association Studies (GWAS), November 2012, Hollywood, California, USA. (The abstract relative to this poster presentation will be reprinting as a supplement associated with the online journal of Cancer Epidemiology, Biomarkers & Prevention.)

**2.** Genetic variation in the in vitro genotoxic response to glycidamide and gene expression of DNA repair genes.
**Pingarilho M**, Oliveira NG, Martins C, Gomes BC, Fernandes AS, Martins V, Silva AR, Rueff J and Gaspar JF
American Association for Cancer research, 2011, Orlando, Flórida, USA.

**3.** Glycidamide-induced cytotoxic and genotoxic effects in human mammary MCF10A cells."

Bandarra S, Gaspar J, **Pingarilho M**, Gil OM, Fernandes AS, Miranda J, Castro M, Rueff J, Oliveira NG.

Society for Free Radical Research - Europe Meeting, September 2010, Oslo, Norway.

**4.** Interindividual Variability in the *In Vitro* Genotoxic Response to Glycidamide: Role of BER and NER Genetic Polymorphisms.

**Pingarilho M**., Oliveira NG, Gomes BC, Martins C, Rueff J and Gaspar JF, Environmental Mutagen Society, 2009, St. Louis, Missouri, USA.

**5.** Evaluation and modulation of the cytotoxic potential of acrylamide and glycidamide

Oliveira N G, Martins C, Vaz S, Martins V, **Pingarilho M**, Rueff J, Gaspar JF

Gordons Research Conference, 2008, Ventura, California, USA.

**6.** Glycidamide-DNA. adducts and sister chromatid exchanges in human lymphocytes exposed to acrylamide and glycidamide.

**Pingarilho M**, Martins C, Oliveira NG, Vaz S, Gamboa da Costa G, Martins V, Marques MM,. Beland FA, Churchwell MI, Doerge DR, Rueff J and Gaspar JF.

98$^{th}$ Annual Meeting of the American Association for Cancer Research, 2007, Los Angeles, California, USA.

**7.** Role of Gluthatione Status on the Cytotoxity induced by Acrilamide and Glycidamide in Mammalian Cells.

Oliveira NG, Martins C, Vaz S, Martins V, **Pingarilho M**, Ruef J and Gaspar J.

13$^{th}$ Annual Meeting of Free Radical Biology & Medicine, November 2006, Denver, Colorado, USA.

**8.** Correlation between DNA adduct formation and cytogenetic damage induction in mammalian cells exposed to acrylamide and glycidamide.

Costa GG, Oliveira NG, Martins C, **Pingarilho M**, Rueff J, Gaspar JF, Martins V, Marques MM,. Beland FA, Churchwell MI and Doerge DR

36$^{th}$ Annual Meeting of the European Environmental Mutagen Society, July 2006, Prague.

**9.** Cytogenetic Damage Induced by Acrylamide and Glycidamide in Mammalian Cells: Correlation with specific Glycidamide DNA-adducts".

Oliveira NG, Martins C, **Pingarilho M,** Costa GG**,** Martins V, Marques MM,. Beland FA, Churchwell MI, Doerge DR, Rueff J and Gaspar JF.

97th Annual Meeting of the American Association for Cancer Research, April 2006, Washington, DC, USA.

Within the scope of this thesis, the following oral communication was presented in a scientific meeting:

In vitro genotoxic response to glycidamide: Role of individual genetic polymorphisms of biotransformation and DNA repair genes.

Gaspar J.F**, Pingarilho M**, Oliveira NG, Martins C, Gomes BC, Fernandes AS, Martins V, Silva AR, Rueff J.

International Conference on Occupational and Environmental Health. Porto, Portugal, Outubro de 2011.

**Table of contents**

**List of Figures**

**List of Tables**

**Abbreviations**

| | |
|---|---|
| 8-OHdG | 8-hydroxy-2'-deoxyguanosine |
| AA | Acrylamide |
| ACEG | Aberrant cells excluding gaps |
| ACIG | Aberrant cells including gaps |
| AFB1 | Aflatoxin B1 |
| AP | Apurinic site |
| BER | Base excision repair |
| BrDU | 5-Bromo-2'deoxyuridine |
| BSO | buthionine sulfoximine |
| CAs | Chromosomal aberrations |
| CAT | Catalase |
| CBMN | Cytokinesis block micronucleus |
| CYP 2E1 | Cytochrome P450 2E1 |
| DMSO | Dimethylsulphoxide |
| DNA | Deoxyribonucleic acid |
| DR | Direct repair |
| EDTA | Ethylynediaminetetractic acid |
| EPHX | Epoxide hydrolase |
| FCS | Fetal calf serum |
| FFQ | Food frequency questionnaire |
| FPG | Fluorescence-plus-Giemsa |
| GA | Glycidamide |
| GC-MS | Gaseous chromatography coupled with mass spectrometry |
| GG-NER | Global genome nucleotide excision repair |
| GP | Genetic olymorphism |
| GPX | Glutathione peroxidase |
| GSH | L-Glutathione reduced |
| GSH-EE | GSH-monoethyl ester |
| GST | Glutathione S-transferase |
| Hb | Hemoglobin |
| HCA | Heterocyclic amine |
| MGHT | Methyltranferase |

| | |
|---|---|
| HPLC-MS/MS | High pressure liquid chromatography coupled with double mass spectrometry |
| HRR | Homologous recombination repair |
| HuGE | Human genome epidemiology |
| IARC | International agency for research on cancer |
| LMP | Low melting point |
| MCB | Monochlorobimane |
| MI | Mitotic index |
| MMR | Mismatch repair |
| MN | Micronucleus |
| mRNA | Messenger ribonucleic acid |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide |
| N1-GA-dA | N1-(2-carboxy-2-hydroxyethyl)-2'-deoxyadenosine |
| N3-GA-Ade | N3-(2-carbamoyl-2-hydroxyethyl)adenine |
| N7-GA-Gua | N7-(2-carbamoyl-2-hydroxyethyl)guanine |
| NAT | N-acetyltransferase |
| NER | Nucleotide Excision repair |
| NHEJ | Non homologous end-joining |
| NMP | Normal melting point |
| NOCs | N-nitroso compounds |
| $O^6$MeGua | $O^6$-methylguanine |
| PAHs | Polycyclic aromatic hdrocarbons |
| PAs | Pyrrolizidine alkaloids |
| PBL | Peripheral blood lymphocytes |
| PBS | phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PHA | Phytohemagglutinin |
| RFLP | Restriction fragment length polymorphism |
| RFU | Relative fluorescence units |
| RNS | Reactive nitrogen specie |
| ROS | Reactive oxygen species |
| RS | Reactive species |
| SCE | Sister Chromatid exchange |

| | |
|---|---|
| SH | Sulfydryl |
| SOD | Superoxide dismutase |
| SSB | Single strand breaks |
| SULT | Sulfotransferase |
| TCR | Transcriptional-coupled repair |
| TTD | Trichothiodystrophy |
| UGT | UDP-glucuronosyltranferase |
| UV | Ultraviolet |
| UVSS | UV-sensitive syndrome |

# Chapter 1

## General introduction

## 1.1. Causes of Cancer

### 1.1.1. Principles

Cancer is a leading cause of death all over the world and is characterized by uncontrolled cellular growth as a result of changes in the genetic and epigenetic information of cells. Each year, tens of millions of people are diagnosed with cancer and more than half of the patients eventually die from it. Moreover, cancer rates could further increase by 50% to 15 million new cases in the year 2020, according to the World Cancer Report [1, 2].

It is known that about 5-10% of all cancers are caused by genetic defects, while 90-95% are caused by environmental factors and lifestyle, including diet (30-35%), tobacco smoking (25-30%) and alcohol (4-6%) [1]. Cancer related with genetic defects can result directly from inherited mutated genes. However, the majority involves alterations or damage accumulation over time of the genetic material within cells [3]. This damage can be caused by both endogenous (internal) and exogenous (environmental) factors, known as important for cancer development [1]. The endogenous causes can be inherited germ line mutation, oxidative stress, generated through normal oxidative metabolism and pathophysiologic states, such as inflammation [1, 3]. There are also several known exogenous factors including tobacco smoking, infectious agents (e.g. viruses, bacteria and parasites), drug intake, radiation, that can damage DNA, both directly by causing breaks in DNA strands and indirectly by interacting with water molecules and generating reactive oxygen species (ROS), industrial chemicals and carcinogenic agents in food and drink that are established as carcinogenic by IARC [3].

Already in 1981 Doll and Peto [4] identified and attempted to quantify the causes of cancer. The factors related with cancer and their relation with the proportions of cancer deaths are listed in next table.

**Table I.1**-Proportions of cancer deaths attributed to various different factors, according to Doll and Peto (from [4]).

| Factor or class of factors | Percent of all cancer deaths | |
|---|---|---|
| | **Best estimate** | **Range of acceptable estimates** |
| Tobacco | 30 | 25-40 |
| Alcohol | 3 | 2-4 |
| Diet | 35 | 10-70 |
| Food additives | <1 | -5-2 [a] |
| Reproductive and sexual behavior | 7 | 1-13 |
| Occupation | 4 | 2-8 |
| Pollution | 2 | <1-5 |
| Industrial products | <1 | <1-2 |
| Medicines and medical procedures | 1 | 0.5-3 |
| Geophysical factors [b] | 3 | 2-4 |
| Infection | 10? | 1-? |
| Unknown | ? | ? |

[a] Allowing for a possibly protective effect of antioxidants and other preservatives.

[b] Only about 1% could reasonably be described as "avoidable". Geophysical factors also cause a much greater proportion of nonfatal cancers (up to 30% of all cancers, depending on ethnic mix and latitude) because of the importance of UV light in causing the relatively nonfatal basal cell and squamous cell carcinomas of sunlight-exposed skin.

According to the previous table, the cause of 97% of all human cancers is explainable and it was estimated that 35% of cancer deaths might be avoidable through changes in diet [4-6]. Diet has long been recognized as potentially important modifiers of cancer risk, beyond that, human beings are often being exposed to carcinogenic factors during their life, some of which are nutritional factors [1]. It is important to note that during their life a human being ingests about 15 tons of dry matter in the form of food [7]. Although many foodstuffs contain genotoxic compounds, the majority of these only occur at low levels, however, multiple genotoxic substances in the same food may result in cumulative or synergistic actions leading to neoplasia in humans [8]. These findings moved attention away from environmental factors such as pollution or viruses or occupational factors, and turned the focus instead onto dietary factors as a major contributor to disease risk [9]. In the same way, through the 1970s and 1980s, many

chemicals from various sources (e.g. environment, occupation and diet) were tested for mutagenic effects with the Ames test and concluded that natural chemicals, present in human diet as complex mixtures may be a more important source of human mutation than environmental or occupational exposure [9].

### 1.1.2. Food contaminants

Food contaminants can be classified as genotoxic and non-genotoxic mutagens according to the mechanistic view of carcinogenesis. Genotoxic agents begin their action at the DNA level, causing DNA damage (gene point mutations, deletions and insertions, recombination, rearrangements and amplifications, as well as chromosomal aberrations). Non-genotoxic agents presumably affect indirectly the cell through tumor promoters, however their modes of action are less defined. These non-genotoxic agents are generally macro-components, e.g. high fat [1].

Genotoxic mutagens are frequently natural products that can be avoided. For instance, through fungal contamination, mycotoxins (e.g. Aflatoxin B1); or anthropogenic chemicals produced through cooking or preserving methods, (e.g. heterocyclic amines (HCAs), polycyclic aromatic hydrocarbons (PAHs), N-nitroso compounds (NOCs) and AA). On the other hand, there are also genotoxic mutagens in natural products that can be present in food and are unavoidable (e.g. Ptaquiloside and Pyrrolizidine alkaloids (PAs)). Furthermore, there are chemicals intentionally added to foods or food coloring, that can act as genotoxic agents, however these cause much less concern, since they are added intentionally [1, 5, 9, 10].

One of the most important genotoxic food carcinogens is aflatoxin B1 (AFB1). This mycotoxin is produced by the mold *Aspargelius flavus*, which grows on poorly (hot and humid climate) stored foods including corn, peanuts and rice [1, 9]. Through epidemiologic studies, AFB1 has shown to increase carcinogenic risk in humans. These toxins proved to be very important liver carcinogens, especially in combination with chronic infection with hepatitis B virus [11-17]. AFB1 initiates its action with metabolic

activation by cytochrome P450, forming an *exo*-8,9-epoxide and subsequent adduct formation producing DNA damage [18, 19].

Other important carcinogenic formed within muscle foods (beef, lamb, and poultry, but also in fish) cooked at high temperatures (e.g. frying, broiling and barbecuing) are the heterocyclic amines [1, 5, 9, 10]. These are formed through a pyrolysis process from amino acids, proteins and creatines of the meat. In humans there is good epidemiologic evidence correlating the consumption of food containing high levels of HCAs and cancer, namely colorectal [14, 20-24], breast [25-27], prostate [14, 28-30] and pancreatic cancers [31-33]. HCA carcinogenesis mechanism encompasses a bioactivation of N-hydroxylation by CYP1A2 and subsequent esterification. The nitrenium ion is likely the ultimate carcinogen, capable of binding guanine at position C8, causing altered DNA sequences with subsequent base substitution, deletion and insertion [1, 34].

PAH compounds are also considered food carcinogens formed during incomplete combustion of organic matter during food processing (smoking, barbecuing and grilling). PAH can also be found in wood fires, automobile exhaust, tobacco smoke and occur as environmental contaminants on food plants (e.g., cereals and vegetable) [1, 9, 35]. In humans there is some evidence of association of dietary PAH exposure with colon cancer [36, 37]. Carcinogenesis mechanism of PAHs is conducted through benzo(a)pirene (BaP) adduct formation, after being activated by CYP1A and CYP1B enzymes. This adduct is associated with site-specific hotspot mutation in p53 tumor suppressor gene [1].

Another important food carcinogen are N-nitroso compounds which can be found in a wide variety of foods, like salted, smoked or dried fish and meat [9]. Moreover, NOCs can be formed *in vivo* during simultaneous ingestion of nitrite or nitrogen oxides and a nitrosable substrate such as a secondary amine [38]. Various types of cancer (lung, liver, kidney, mammary gland, among others) have been observed and related to NOCs in humans [1, 39, 40]. The common carcinogenic mechanism of N-nitrosamines requires metabolic activation through hydroxylation. This is catalyzed mainly by CYP2E1, but other cytochrome P450 isoforms including CYP2A6 have been implicated. N-nitrosodimethylamine undergoes enzymatic hydroxylation and

subsequent hydrolysis to aldehyde and monoalkylnytrosamine that rearranges and releases a carbocation that is reactive toward DNA bases. [1, 38].

Acrylamide is another important carcinogen formed through cooking, identified in starch-based foods such as potato chips and French fries cooked using high temperature deep-frying, grill and baking methods [35]. Acrylamide is clearly an animal carcinogen and a neurotoxin. However, extrapolation of effects in cell systems and in animals to effects in humans has been controversial [9]. This compound is the main focus of the present study and because of its importance in the development of this thesis, a more explanatory chapter will be developed ahead (chapter point 1.5.).

Despite the great importance attributed to the existence of food genotoxic agents, the relevance of the absence of some dietary components should also been taken into account, especially micronutrients, that can be related with increased cancer risk. Folate deficiency is well known as one of the most common vitamin deficiencies, which contributes to chromosomal instability and may increase susceptibility to radiation-induced DNA damage [9, 41]. Folate deficiency may contribute to carcinogenesis by causing DNA hypo-methylation and proto-oncogene activation or by inducing uracil misincorporation during DNA synthesis [41]. Another, equally important deficiency is the lack of selenium, which has been linked with increased cancer risk. Some studies reported selenium supplementation as protective against the development of cancer at numerous sites including prostate, colon, and lung. Although the mechanisms of chemoprevention by selenium remain unclear, enhanced protection against oxidative stress may be involved [42, 43].

As a general conclusion, one can say that conventional epidemiology can show association between cancer and some types of food, and/or with cooking process. However, these are not constitutive proofs of cause and effect. It is difficult, if not impossible, to attribute such results with certainty to any specific compound, since food is a complex mixture [9]. Many food components have already genotoxic potential and more can be produced endogenously during digestion [5]. There is increasing evidence that consumption of some foods, like fresh fruits and vegetables may decrease the risk of cancer. In the same way, a number of plant constituents have been shown to have the potential to inhibit various stages of the carcinogenic process [5]. Consequently, the risk of cancer related with nutrition outcome from an imbalance of carcinogenesis and anti-

carcinogenesis process [1]. Nevertheless, the role of food and nutrition in the modification of the cancer process is very complex [5].

## 1.2. Biomarkers of genetic DNA damage

### 1.2.1. Principles

The National Academy of Sciences defines a biomarker as a xenobiotically induced alteration in cellular or biochemical components or processes, structures or functions that is measurable in a biological system or sample, this means that biomarkers are observable endpoints that indicate events in the processes leading to disease [44, 45].

Biomarkers are becoming increasingly important in toxicology and human health and many research groups are carrying out studies to develop biomarkers of exposure to chemicals and apply these for human biomonitoring [46]. Biological monitoring has advantages over environmental monitoring because it measures the internal dose of a compound. However, is important take into account the inter-individual differences in absorption, bioavailability, excretion and DNA repair [44].

Biomarkers used in human health studies are typically divided into three classes: biomarkers of exposure, effect and susceptibility (Fig. 1.1), depending on their toxicological significance, whose concepts will be developed later.

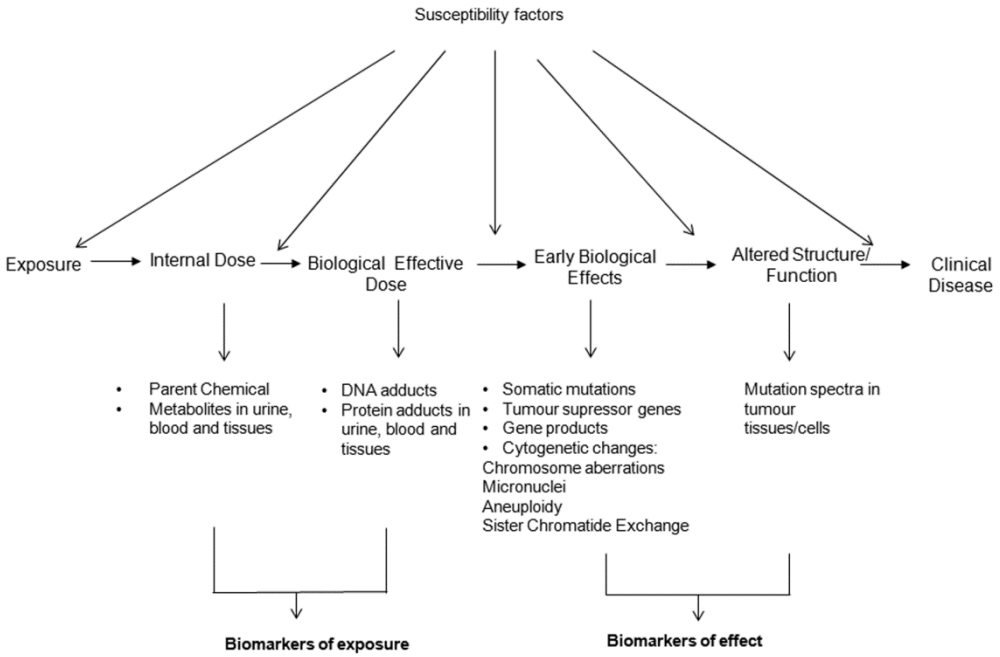



**Fig. 1.1-** Relation of events and biomarker classification (adapted from [46]).

Susceptible individuals could be identified by biomonitoring and molecular epidemiology, particularly those suffering a combination of high risk factors, namely a high level of exposure to chemicals, inherited cancer predisposing genes and a deficiency of protective factors. Individual susceptibility factors can influence all the stages between exposure and the onset of disease (Fig. 1.1) [46].

### 1.2.2. Biomarker of exposure

A biomarker of exposure is a chemical, its metabolite or the product of an interaction between a chemical and some target molecule or macromolecule that is measured in a compartment or a fluid of an organism [45]. It involves measurements of the internal dose by chemical analysis of the parent compound, metabolites or DNA or protein adducts in body fluids or excreta such as blood, urine and exhaled air [44, 47].

Biomarkers of exposure can be divided into biomarkers of internal dose and biomarkers of biological effective dose [46, 48].

#### 1.2.2.1. Biomarkers of internal dose

Biomarkers of internal dose are indicative of the occurrence and extent of exposure of the organism [48]. These markers indicate the actual exposure to a particular compound that occurred by measuring the compound or its metabolite(s) in body fluids. However these biomarkers do not reveal to what extent the metabolized agent has affected the target tissue or cells [48]. One example is the measurement of the excretion of 1-hydroxypyrene, an urinary metabolite that is widely used for measurement of exposure of PAHs. The excretion of this metabolite was found to correlate well with PAHs exposure. Another example is mercapturic acids in urine that have also been used for monitoring exposure to a number of specific chemicals, for example epichlorohydrin and styrene [46].

#### 1.2.2.2. Biomarkers of effective dose

Biomarkers of effective dose are indicative of the extend of exposure of the target molecule, structure or cell [48]. These biomarkers included the measurement of adducts formed by the reaction products of alkylation of endogenous or exogenous chemicals compounds, often called alkylating agents, and cellular macromolecules, such as proteins and DNA [49, 50], giving rise to hemoglobin (Hb) and DNA adducts. The alkylation occurs between the nucleophilic atoms (nitrogen, oxygen, or sulfur) within the biomolecule and an electrophilic atom in the reactive molecule [49]. This is especially useful, since represents the dose that has escaped the detoxification process and that has reached the macromolecule [46].

*DNA adducts*

It is well known that genotoxic carcinogens-like alkylating agents or epoxides initiate tumorigenesis by reacting with nucleophilic sites of DNA and by generating DNA adducts [51]. Besides the DNA adducts that can be formed from alkylating agents, numerous DNA adducts are also formed endogenously, for example from the methylating factor S-adenosylmethionine or by oxidative metabolism that produces ROS [49].

The use of DNA adducts as biomarker have disadvantages, because DNA from susceptible human tissues is not readily accessible in large amounts and DNA adducts are susceptible to repair and at different rates depending on the tissue, cell type and DNA region [44]. Moreover, the stability of DNA adducts is a complex issue in investigation, because some adducts are naturally chemically unstable (e.g. guanine N7 and adenine N3 adducts) generating repairable apurinic sites (AP) on DNA. It is also important to note that DNA adducts can suffer enzymatic repair [49]. The formation of adducts by the reaction of chemicals with DNA is thought to be the critical step for the initiation of carcinogenesis [50, 52, 53]. Up to now DNA adducts do not allow a quantitative estimate of cancer risk. However, the occurrence of DNA adducts show at least an elevated cancer risk [52]. DNA adducts not only represent an exposure that already occurred, but they also imply a potential for significant biological consequences, e.g. mutations [53].

DNA adducts analysis started in the beginning of the 1980s when Randerath *et al* developed the $^{32}$P-postlabelling analysis technique [46, 49]. Later, another method of analysis of DNA adducts that became popular was the reversed phase HPLC-MS/MS. Nowadays, tandem mass spectrometry, particularly if combined with HPLC, is currently the recommended detection technique [49].

*Protein adducts*

Protein alkylation products are stable *in vivo* and thus are excellent targets for biomonitoring purposes. The most commonly used molecules are hemoglobin and albumin, because these molecules can be obtained in an easy way from blood samples. The most commonly studied alkylation site on hemoglobin is the N-terminal valine,

however sulfhydryl group of cysteine and nitrogen of histidin are also preferred sites of binding [49, 52].

Protein adducts can be regarded as an integrative exposure methods. One good example is that hemoglobin adducts are considered good biomarkers to measure the cumulative internal dose due to repeated exposures, since red blood cells live for as long as 4 months in humans [46, 49, 54]. These type of adducts are chemically stable and they are not prone to repair mechanisms [46, 49, 52]. In contrast, albumin adducts have a shorter lifetime in blood of about 20 days and therefore reflect a more limited period of exposure [46].

The important role of protein adducts were highlighted in 2002 by a study were high levels of acrylamide protein adducts were found in occupational settings [49]. However, there are several compounds including PAHs, HCAs, aromatic amines, micotoxins and chemotherapeutic agents, among others that forms Hb-adducts [44].

The protein adducts analysis was developed by Enrenberg's group in Stockholm based on the hemoglobin molecule [46]. The most widely applied and most successful procedure is through the modified Edman degradation of globin protein. In this method, globin is precipitated from red blood cells and the valine terminal of hemoglobin is cleaved. Subsequently, adducts are analyzed by GC-MS [46, 49, 52].

### 1.2.3. Biomarkers of effects

A biomarker of effect is a measurable biochemical, structural, functional, behavioral or any other kind of alteration in an organism that, according to its magnitude, can be associated with an established or potential health impairment or disease [45]. These include well-established biomarkers for chromosome damage measured by micronuclei, chromosome aberrations, sister chromatid exchanges and comet assay.

#### 1.2.3.1. Micronuclei (MN)

Measurement of micronuclei frequency in human lymphocytes is one of the most commonly used methods for measuring DNA damage in human populations exposed to genotoxic agents [55, 56]. This assay has been also successfully applied to identify occupational, dietary and genetic factors that have a significant impact on genome stability [55].

Micronucleus is originated from chromosome fragments or whole chromosomes that fail to engage with the mitotic spindle and therefore lag behind when the cell divides [56]. The formation of MN in dividing cells is the result of chromosome breakage (clastogenesis) due to unrepaired or mis-repaired DNA lesions, or chromosome mal-segregation (aneugenesis) due to mitotic malfunction [55, 57]. The most widely used test for the detection of MN is based on the use of cytochalasin B, a fungal metabolite that inhibits cytokinesis, being this assay named the cytokinesis-block micronucleus (CBMN) test [57].

Compared to other cytogenetic assays, quantification of MN, using the CBMN assay, confer several advantages, including high reliability and low cost of the technique, no requirement for metaphase cells and reliable identification of cells that have completed only one nuclear division, which prevents confounding effects [55, 56].

According to Bonassi *et al* [55] there is an association between MN induction and cancer development. This association was also evident in a cohort study done by the Human MicroNucleus project, where there are significant evidences in all cohorts for all major cancer sites, especially urogenital and gastrointestinal cancers. This study provided valuable evidence that MN frequency in PBL is predictive of cancer risk, suggesting that increased MN formation is associated with early events in carcinogenesis [58].

#### 1.2.3.2. Chromosomal aberrations (CAs)

CAs has been used as a biomarker of chromosomal damage and genome instability and represent the most extensively used and validated biomarker in populations exposed to genotoxic agents [56, 57, 59].

Chromosomal aberrations are changes in normal chromosome structure (structural aberrations) or number (numerical aberrations) that can occur spontaneously or as a result of chemical/radiation treatment. Structural CAs may be induced by direct DNA breakage, by replication on a damaged DNA template, by inhibition of DNA synthesis and by other mechanisms (e.g. topoisomerase II inhibitors) [60]. Numerical CAs refers to changes in normal chromosome number (i.e. aneuploidy, polyploidy) which occur due to abnormal chromosome segregation; they may arise either spontaneously or as a result of aneugen treatment [60]. CAs are evaluated in stimulated peripheral blood lymphocytes arrested at metaphase and stained, usually by the Giemsa band technique [57].

An increased frequency of CAs in circulating lymphocytes is generally considered indicative of increased cancer risk for those exposed to DNA damaging agents [56, 61]. Moreover data obtained from both studies carried out by Hagmar et al. [62] and Bonassi and Abbondandolo [63], indicated that the frequency of CAs in peripheral blood lymphocytes is a relevant biomarker for cancer risk in humans, reflecting both early biological effects of exposure to genotoxic carcinogens and individual cancer susceptibility [56]. In spite of the excellent sensibility of this technique and proved predictive value regarding cancer risk, the detection of chromosomal aberrations is technically demanding and a slow process [57].

#### 1.2.3.3. Sister chromatid exchange (SCE)

SCE is the process whereby the sister chromatid effectively break and rejoin with one another, physically exchanging regions. SCEs are formed during the S phase of the cell cycle and can be induced by UV light and a large number of genotoxic chemicals, especially those chemicals that are S-phase-dependent clastogens [64, 65]. They can be visualized in cultured cells when division is induced in the presence of 5-bromodeoxyuridine (BrdU) [57].

According to Suspiro and Prista [57] there is some uncertainty regarding the significance of increased SCE frequency with regard to cancer risk. Norppa *et al* (2006) reviewed some of the results of the European collaborative project (Cancer Risk Biomarkers) and suggest that the association between frequencies of SCEs and cancer

risk may be difficult to predict [66]. They also observed that the frequencies of SCEs are heavily affected by technical variation, which makes it difficult to define a high SCE level when data from a number of studies are combined. However, SCEs are known to be increased by exposure to various genotoxic carcinogens and seem to reflect the repair of DNA lesions by homologous recombination [66]. SCE assay is well-known for its sensitivity to detect DNA damage induced by chemical genotoxicants.

#### 1.2.3.4. Comet assay

The comet assay, also known as single cell gel electrophoresis, is a versatile and sensitive method for measuring DNA damage. This technique has become very popular for the assessment of DNA damage with applications in genotoxicity testing, human biomonitoring and molecular epidemiology, ecotoxicology, as well as in research in DNA damage and repair [67]. Under alkaline (pH>13) conditions, the assay can detect single and double stranded breaks, incomplete labile sites, alkali labile sites, and also possibly both DNA-protein and DNA-DNA cross-links in eukaryotic cells [68-70].

The comet assay consists of a single cell suspension embedded in agarose and layered onto a microscope slide, after lysis to deliberate DNA content and electrophoresed under alkaline conditions. The product can be visualized after staining with a suitable dye [52, 57, 71]. This type of test as many advantages, namely high sensitivity for detecting low levels of DNA damage, requirement of small number of viable cells per sample, the simplicity, low cost and short time of test performance [57, 71]. However is important to note that there is a wide variability of the comet data since the basal level of DNA damage is influenced by a variety of factors such as lifestyle, diet, infections, medication, air pollution, season, climate or exercise [52].

The significance of comet assay as a marker of increased cancer risk remains unclear [57]. Comet assay can be considered, for the time being, a biomarker of exposure rather than a biomarker of effect, due to the lack of prospective studies demonstrating an increased cancer risk [57, 71]. It should however been mentioned that the comet assay is actually an emerging tool to properly assess primary DNA damage either *in vitro* or *in vivo.*

### 1.2.4. Biomarker of susceptibility

A biomarker of susceptibility may be defined as an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a chemical [45]. They serve as indicators of particular sensitivity of individuals to the effect of a xenobiotic or to the effects of a group of such compounds. They can be genetic markers that include alterations in chromosomal structures, genetic polymorphisms, among others [44].

It is generally agreed that genetic polymorphisms (GP) are associated with most common disorders with a genetic component such as cancer. However, the complex metabolism of these compounds involving different polymorphic genes and also different DNA repair polymorphic genes could in association modulate the individual risk factor for this kind of disease [61].

It is normally accepted that the biotransformation of xenobiotic compounds including drugs involved mainly two Phases I and II. Phase I reaction include transformation of a parent compound to more polar metabolite(s). For example, phase I reactions includes *N*- and *O*-dealkylation, aliphatic and aromatic hydrolylation, *N*- and *S*-oxidation and deamination. The main enzymes in this phase are cytochrome P450 (CYPs) performing mainly hydroxylations and hence acting as monooxygenases, dioxygenases and hydrolases [72].

Phase II enzymes play also an important role in the biotransformation of endogenous compounds as xenobiotics to more easily excretable forms. The purpose of phase II biotransformation is to perform conjugating reactions. These include glucuronidation, sulfation, methylation, acetylation, glutathione and amino acid conjugation. In general, the respective conjugates are more hydrophilic than the parent compounds. Phase II drug metabolizing enzymes are mostly transferases and include: UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), N-acetyltransferases (NATs), glutathione S-transferases (GSTs) and epoxide hydrolase (EPHX) [44, 72-74].

In general the actions of phase I and phase II enzymes render susceptible compounds more soluble and more readily excreted and ought to reduce genetic damage and cancer risk with several exceptions. It is important to note that some authors

consider epoxide hydrolase as a phase II enzyme [73, 74] while others consider the same as a phase I enzyme [72].

#### 1.2.4.1. Main Metabolism/Detoxification polymorphisms

*Cytochrome P450 family (CYPs)*

The family of CYPs is involved in the metabolism of several xenobiotics, biosynthesis of steroids, lipids, vitamins and natural products. [44, 75]. The CYPs are enzymes which catalyze the insertion of one atom of molecular oxygen into a substrate [61]. The liver generally expresses the highest CYP activity, but all tissues express the enzymes in a tissue-specific manner [76]. Some of the enzymes of CYP family will be discussed below.

CYP1A is one of the major Phase I enzymes responsible for the metabolic activation of PAHs (e.g. benzo[a]pyrene), one of the main carcinogens found in cigarette smoke and environmental pollution [61, 77, 78]. Previous studies have described several polymorphisms in the *CYP1A1* gene (*CYP1A1*2A* and *CYP1A1*2C* for example) [79, 80]. In relation to CYP1A1 an association between this polymorphism and cancer risk, namely lung cancer [81], susceptibility in childhood acute lymphoblastic leukemia [82] and colorectal cancer [83] was found. On the other hand, no association was found, specifically with renal cell carcinoma [79, 84] and esophageal cancer [85].

Another example is CYP2E1 that plays an important role in the activation of a variety of carcinogens, including nitrosamines, some components of tobacco smoke, and many organic chloride and non-chloride solvents, including benzene and also AA [61, 78]. This enzyme may be induced by ethanol, and thus alcohol intake may influence carcinogenesis by exposure to carcinogens activated by CYP2E1 [61]. This enzyme is constitutively expressed in the liver and in many other tissues and is of clinical and toxicological importance [86].

Concerning the correlation of *CYP2E1* and cancer risk, some studies observed that *CYP2E1* polymorphism may affect the susceptibility to lung cancer [87] and of esophageal squamous cell carcinoma [78].

*Microsomal epoxide hydrolase polymorphisms*

The EPHX1catalyzes the hydrolysis of reactive epoxides to their corresponding dihydrodriols, playing an important role in detoxification of epoxides [78, 88]. This irreversible reaction produces metabolites, which are more water soluble, less reactive, and readily conjugated and excreted [61]. Although EPHX is considered a detoxifying enzyme, the dihydrodiol deriving from PAHs may be further transformed by CYP into more reactive species, an example is dihidrodiol epoxides, that are the most mutagenic and carcinogenic of PAHs metabolites [61]. EPHX1 is expressed in all tissues studied, including white blood cells [88].

For *EPHX1* SNP and like an example of cancer risk association, in white populations, the high-activity (variant) genotype of *EPHX1* polymorphism at exon 4 was associated with a modest increase in risk of lung cancer, while the low-activity of *EPHX1* polymorphism at exon 3 was associated with decreased risk of lung cancer [89, 90].

*Glutathione S-transferases (GSTs) polymorphisms*

GSTs, one of the major phase II detoxification enzymes are involved in the metabolism of xenobiotics and play an important role in cellular process against oxidative stress [72].

GSTs play a major role in the detoxification of epoxides derived from PAHs and alfa-beta unsaturated ketones. Moreover, a number of endogenous compounds such as prostaglandins and steroids are metabolized via glutathione conjugation [72-74, 91].

Human GST enzymes belong to five different classes designated by Alpha, Gamma, Mu, Pi and Theta, with their isoenzyme type designed by Arabic numerals. Several types of allelic variations have been identified in the class Alpha, Mu, Pi and Tetha gene families [73, 74]. Overall, individuals lacking *GSTM1*, *GSTT1* and *GSTP1*

genes have a higher incidence of bladder, breast, colorectal, head/neck and lung cancer. Loss of these genes has also been found to increase susceptibility to asthma and allergies, atherosclerosis and rheumatoid arthritis [72].

GSTT1 enzyme is expressed mainly in the liver and kidney, but also in red blood cells and is involved in the metabolism of several important epoxides, such as methylene chloride and ethylene oxide [74]. Overall, epidemiologic studies do not show any clear association between the *GSTT1* null genotype and cancer development [73, 74, 76].

GSTM1 enzyme is expressed in many organs including liver, testis, adrenals and white blood cells and metabolizes epoxides such as styrene 7,8-oxide and the ultimate form of aflatoxin B1 [74, 76]. *GSTM1* null genotype was not associated with risk of oral and lung cancer in Caucasians [92, 93], however it was associated with an increased risk of sporadic colorectal cancer [94].

The GSTP1 is widely expressed in tissues and is the major enzyme in the blood (white and red cells). Polymorphisms in *GSTP1* have been associated with a reduction in enzymatic activity toward several substrates, including both chemotherapy agents (such as cisplatin, a common agent used in lung cancer treatment) and carcinogens found in tobacco smoke [95]. The association between *GSTP1* and lung cancer risk was examined by Cote *et al* that found no significant association between this type of cancer and the *GSTP1* exon 5 polymorphism [95]. However, *GSTP1* Ile105Val appears to be associated with a modest increase in the risk of bladder cancer [96]. Moreover, Ramos *et al* [97] suggested a possible role of *GSTP1* on the modulation of the genotoxicity induced by Doxorubicin.

GSTA2 belongs to the Alpha class of GSTs that are strongly expressed in liver, kidney and adrenal tissue. The Alpha class has commonly been described as one of the most versatile GST families, since it is responsible for GSH conjugation of compounds such as bilirubin, bile acids and penicillin, thyroid and steroid hormones, allowing their solubilisation and storage in the liver [98, 99]. Since the Alpha family is involved in a wide range of roles that include steroid biosynthesis and providing protection against alkylating agents, polymorphic variations in these genes could be responsible for physiological consequences that could alter the susceptibility to disease and drug response [91, 99]. Two members of this class, GSTA1 and GSTA2 catalyze the GSH

conjugation of a wide variety of electrophiles, possess glutathione-dependent steroid isomerase activity, and glutathione-dependent peroxidase activity [100]. The *GSTA2* gene is believed to represent a major line of defence against oxidative stress [99]. No association was observed between individual *GSTA2* polymorphisms and individual susceptibility, namely to breast [98, 101], colorectal and prostate cancer [102]. However, the variant allele of this polymorphism seems to be associated with an increased risk of lung cancer [103].

#### 1.2.4.2. DNA repair enzymes polymorphisms

As abovementioned, a wide variety of DNA damage may be induced by normal endogenous metabolic processes or by environmental carcinogens. If not repaired, such damage can lead to gene mutations and genomic instability, which in turn may cause malignant transformation of cells. The individual response to DNA damage induced by xenobiotics depends largely on the efficiency of DNA repair mechanisms. Normal function of DNA repair enzymes is essential for the removal of damage. It has been shown that reduced DNA repair is associated with increased risk of cancer.

These repair mechanisms and respective enzymes, due to their importance in the present work, will be discussed in the next section entitled DNA damage.

## 1.3. DNA Damage

### 1.3.1. Principles

DNA damage is a relatively common event in the life of a cell and may lead to mutation, cancer and cellular or organismic death [104]. The genome is inherently unstable due to spontaneous chemical reactions, and its fidelity is compromised due to very low but significant replication errors [105]. Moreover, the genomes of all organisms are continuously exposed to a wide variety of insults, responsible for the lesions that arise in DNA (Figure 1.2). The most common insults are environmental agents, such as the ultraviolet (UV) component of sunlight, ionizing radiation and numerous genotoxic chemicals. Products of normal cellular metabolism that include ROS (superoxide anions, hydroxyl radicals and hydrogen peroxide) derived from oxidative respiration and products of lipid peroxidation cannot be forgotten. Finally, under physiological conditions, some chemical bond in DNA tends to spontaneously break, leading non-instructive abasic sites from hydrolysis of nucleotide residues [106].

The most common types of DNA damage and their sources are summarized in Figure 1.2.

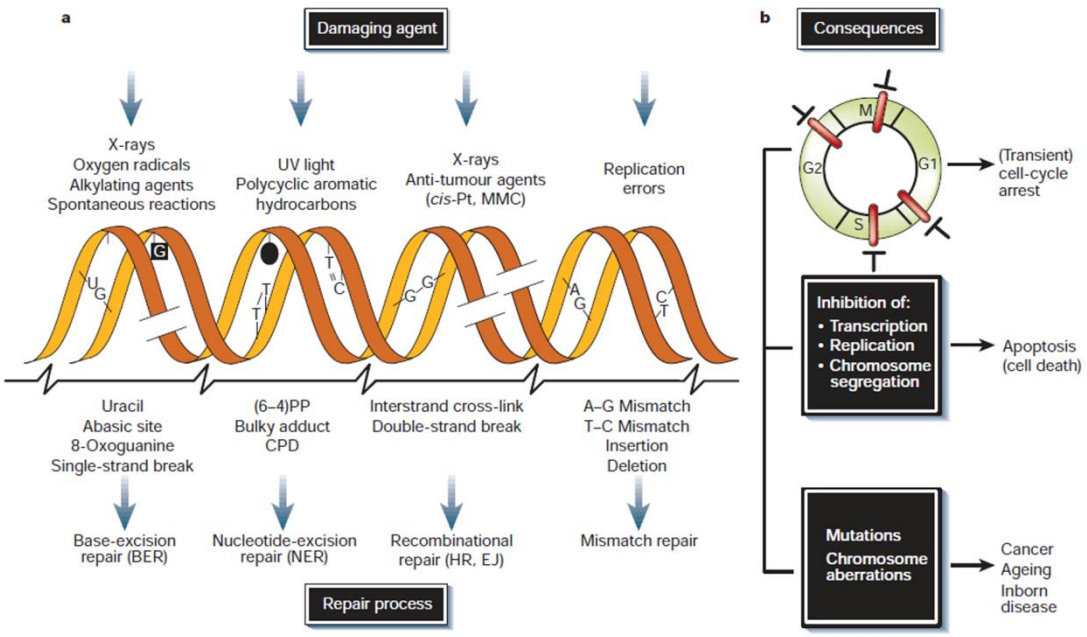



**Fig. 1.2–** DNA damage, repair mechanisms and consequences (from [106]).

These endogenous and exogenous insults promote several DNA lesions, such as single and double strand breaks, abasic sites and the formation of DNA adducts (Fig.1.2). These DNA lesions need to be repaired in order to a proper functioning of the cell, by DNA repair systems discussed in next section point.

### 1.3.2. DNA Damage Repair System

The cells need a multiple DNA repair pathways to avoid the broad DNA damaging agents which are responsible for different types of DNA lesions. These pathways include: (a) direct repair of alkyl adducts by $O^6$-alkylguanine DNA alkyltransferase, (b) repair of base damage and single strand breaks by base-excision repair (BER), (c) repair of bulky adducts by nucleotide-excision repair (NER), (d) repair of double strand breaks by homologous recombination repair (HRR) and non-homologous end joining (NHEJ) and (f) repair of mismatches and insertion/deletion loops by DNA mismatch repair (MMR) [106, 107].

#### 1.3.2.1. Direct repair (DR)

Direct repair is involved in DNA damage repair due to alkylating agents. There are two different mechanisms of direct repair in the majority of organisms: the photoreversal of UV-induced pyrimidine dimmers and the removal of the $O^6$-methyl group from $O^6$-methylguanine ($O^6$MeGua) in DNA [104]. In mammals, the only known DR pathway is the last one, that is comprised of a single protein (and thus a single gene), termed $O^6$-methylguanine ($O^6$-MeGua) DNA methyltransferase (MGMT). This protein transfers the alkyl group at the $O^6$ position of guanine to a cysteine residue within its active site, leading to the direct restoration of the natural chemical composition of DNA without the need for genomic "reconstruction". However, this repair event leads to the irreversible inactivation of the MGMT protein and thus has often been referred to as a "suicide" reaction [104, 108]. Deficiencies in MGMT can

lead to an increase in mutations, in part because $O^6$-*MeG* mispairs with thymine during DNA replication [108].

#### 1.3.2.2. Base excision repair (BER)

Base excision repair commonly deals with small chemical alterations of bases that may or may not block the transcription and replication, although they frequently miscode. BER is particularly important for preventing mutagenesis and the main lesions subjected to BER are oxidized DNA bases, arising spontaneously within the cell during inflammatory responses, or from exposure to endogenous agents, including ionizing radiation and long-wave UV light [106, 109]. Lesions for this repair process affect only one of the DNA strands. BER is known as the main guardian against DNA damage, commonly due to cellular metabolism, including that resulting from reactive oxygen species, methylation, deamination and hydroxylation [106] and is responsible for removing DNA-damaged bases, which can be recognized by specific enzymes, the DNA glycosylases [109].

BER presents two optional repair patches depending on the initial events in base removal, the short patch, the dominant mode, when one single nucleotide is removed and the long patch when 2-10 nucleotides are removed [104].

Base excision repair is initiated by a DNA glycosylase which catalyzes the hydrolysis of the N-glycosyl bond between the base and the sugar phosphate backbone forming an abasic site (AP) in the DNA [106, 110]. The resulting abasic site can also occur spontaneously by hydrolysis [106] .

There are different DNA glycosylases that recognize oxidized/reduced bases, alkylated (usually methylated) bases, deaminated bases (e.g. uracil, xanthine), or bases mismatches. In BER, the damaged base is removed by different DNA glycosylases (depending on the damage) and APE1 endonuclease [111]. Two classes of DNA glycosylases have been identified, some DNA glycosylases catalyses only the hydrolytic removal of the base to form apurinic/apyrimidinic (AP) site, whereas others remove the base and subsequently cleave off the base by a lyase mechanism and catalyze a subsequent AP lyase reaction [104]. The initiating glycosylase dictates the downstream repair events.

When excision is done by a glycosylase without associated AP lyase, a free base is released and an AP site is formed in DNA. The AP site is cleaved by a 5' AP endonuclease (APE1), generating a 3'-hydroxyl group and a 5'-deoxyribose phosphate moiety [110]. The later one can be removed by DNA polymerase β (Pol β), leaving a one-nucleotide gap in DNA, which will be filled by DNA Pol β (short patch BER). Alternatively, when the AP site is formed by spontaneous hydrolysis, the 5'-deoxyribose phosphate can be displaced from its complementary DNA which may involve Pol δ/ε (DNA polymerase δ/ε) and PCNA (proliferating cell nuclear antigen) for repair synthesis (2-10 bases) as well as the FEN1 endonuclease to remove the displaced DNA flap [106, 110]. If BER is initiated by a glycosylase/ AP lyase the resulting AP site is cleaved at its 3' by the glycosylase-associated AP lyase activity immediately after the base excision. The 3' baseless sugar phosphate at the DNA nick can subsequently be removed by the 5' AP endonuclease, generating one nucleotide gap [110]. BER is completed by a DNA ligation step, involving DNA ligase III-XRCC1 complex and DNA ligase I [106, 110].

Up to now a mutation in germ line gene *MUTYH* (*MYH*) is associated with a predisposition to multiple colorectal adenomas, known as MYH polyps and is described as an autosomal recessive [112-114]

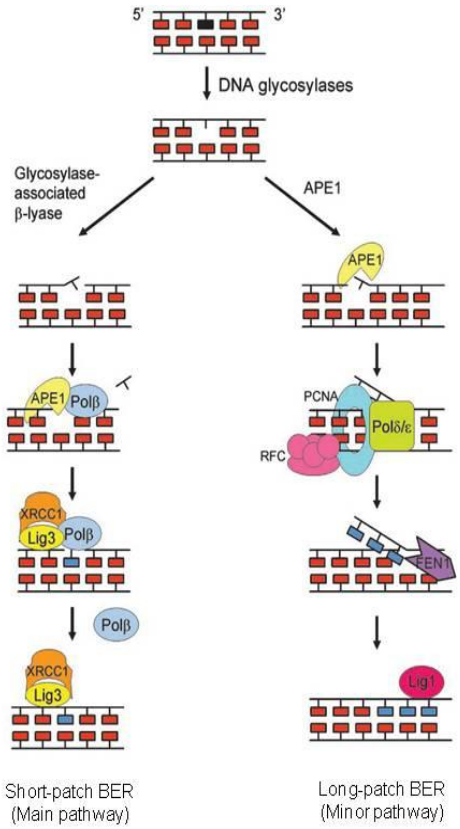



**Fig. 1.3**–Base excision repair mechanisms in mammalian cells (from [104]).

### 1.3.2.3. Nucleotide excision repair (NER)

Nucleotide excision repair deals with the wide class of helix-distorting lesions that interfere with base pairing and generally obstruct transcription and normal replication. Most NER lesions arise from exogenous sources [106]. NER is the most important repair system to remove bulky DNA lesions that can be caused by UV radiation and large chemical adducts generated from exposure to aflatoxine, benzo[a]pyrene and other genotoxic agents [109, 111]. Of all repair systems, NER is the most versatile in terms of lesion recognition. Two NER sub-pathways exist with partly distinct substrate specificity: global genome NER (GG-NER) and transcriptional-coupled repair (TCR). GG-NER scans genome constantly and removes lesions from the non-transcribed domains of the genome and the non-transcribed strand of transcribed regions. TCR removes different RNA-polymerase-blocking lesions from the transcribed strand of active genes and is thought to be focuses on damage that blocks elongating RNA polymerases [106, 109, 111].

NER is mechanistically complex, involving more than 20 proteins in the repair pathway (Figure 1.4) and can be divided into five distinct steps: (a) damage recognition, (b) dual incisions bracketing the lesion, (c) excision, (d) repair synthesis to fill in the resulting gap and (e) DNA ligation [104, 110].

In GG-NER, the DNA lesions are recognized by the XPC-HR23B complex, whereas in TCR the lesions are recognized by two specific factors: CSB and CSA [106]. The subsequent stages of GG-NER and TCR may be identical. After recognition of the lesion, the transcript factor TFIIH, consisting of seven different proteins, is recruited to the site of DNA damage and is the responsible for unwinding DNA around the lesion [109]. After the formation of an open complex the excision of the lesion is carried out by dual incisions at defined positions flanking the DNA damage. XPG and XPF-ERCC1 are respectively responsible for the cleavage in 3' and 5′ of the borders of the opened stretch only in the damage strand, generating a 24-32 base oligonucleotide containing the injury [106, 109]. The arising DNA gap is filled in by Polδ and Polε with the aid of replication accessory proteins PCNA and RFC and sealed by DNA ligase I [104, 109]. Cells defective in NER belong to different complementation groups and UV-hypersensitive disorders, such as Xeroderma Pigmentosum, Cockayne syndrome, trichothiodystrophy (TTD) and UV-sensitive syndrome (UVSS), all characterized by photosensitivity, predisposition to cancer and neurological degeneration in some cases [106, 109, 110]. The Cockayne syndrome is thought to be directly related with defects in TCR [109].

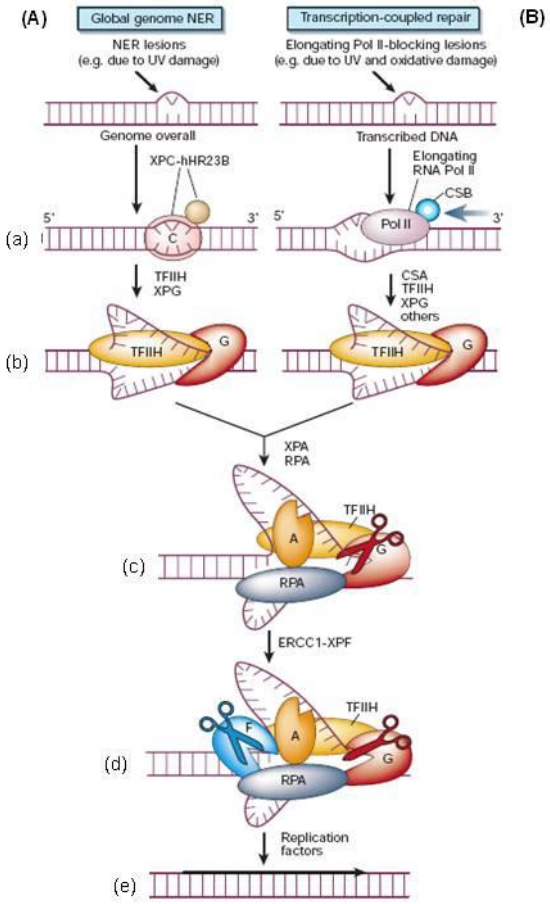



**Fig. 1.4**- Mechanism of nucleotide excision repair (NER). A- global genome repair and B- transcription-coupled repair (from [106]).

### 1.3.2.4. Double strand breaks

Double strand breaks (DSBs) are highly potent inducers of genotoxic effects and cell death [109]. In DSBs both strands are affected and the cell has to know which ends belongs together what is a very difficult task given the size of the mammalian genome [106]. Because both strand of the DNA helix are broken, chromosomal fragmentation, translocations and deletions can easily occur and rapid repair is crucial [111]. DNA DSBs can be caused by ionizing radiation, ROS and chemotherapeutic drugs and can arise during replication of a single strand break [106, 111].

There are two possible pathways for the repair of DNA double strand breaks, the homologous recombination repair (HRR) and non homologous end joining (NHEJ). The two main differences between these pathways are the requirement for extensive DNA

homology on the sister chromatid in HRR and the accuracy of repair [111]. When after replication, a second identical copy is available, homologous recombination seems to be preferred, otherwise cells rely on end joining, which is more error-prone [106]. The usage of NHEJ and HRR also depends on the phase of the cell cycle. NHEJ occurs mainly in G0 and G1 or terminally differentiated cells, whereas HRR occurs during the late S or G2 phase [109, 111].

The NHEJ system is initiated by the biding of a heterodimeric complex consisting of the Ku70 and Ku80 proteins to the damaged DNA. Following DNA biding, the Ku-heterodimer associates with the catalytic subunit of DNA-PK, which is activated by interaction with single strand DNA at the site of DSB and display Ser/Thr kinase activity. The XRCC4-ligase IV complex is the responsible for the link of duplex DNA molecules with complementary but non ligatable ends [104, 109]. The HRR is more complex, since DSBs generated by mutagenic agents needs to be processed first. This process (Figure 1.5) has three steps: (a) strand invasion, (b) branch migration and (c) Holliday junction formation. This repair process is initiated by a nucleolytic resection of the DSB in the 5'-3' direction by the MRE11-Rad50-NBS1 complex which displaysexonuclease, endonuclease and helicase activity. The resulting 3' single-stranded DNA is thereafter bound by a heptameric ring complex formed by Rad 52 proteins. After DSB recognition and strand exchange performed by Rad proteins, the resulting structures are resolved according to the model of Holliday [104, 109].

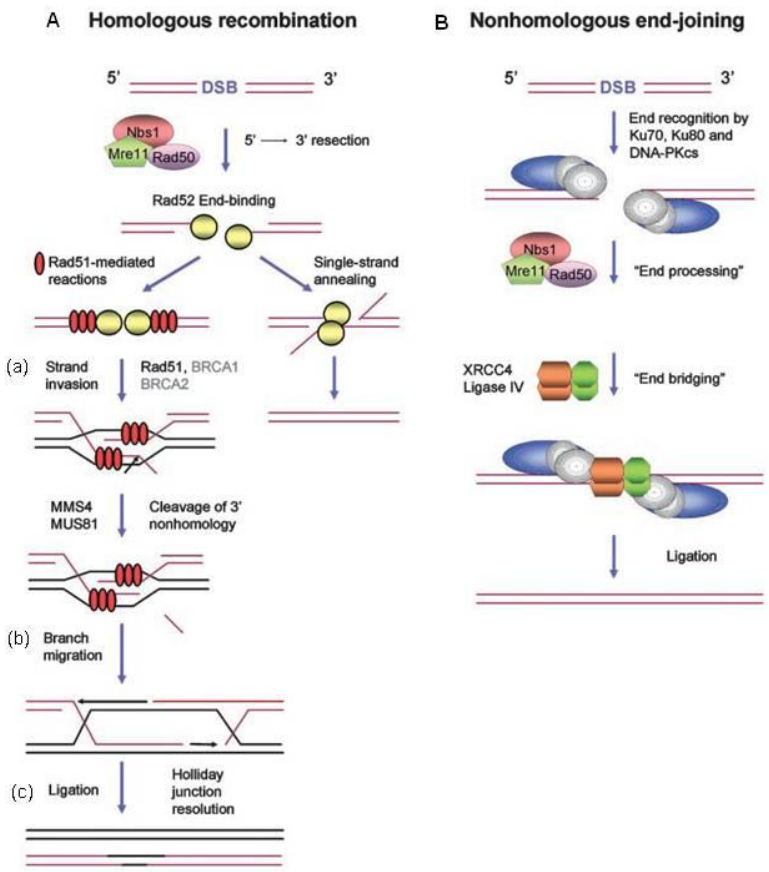



**Fig. 1.5-**Double strand break/recombinational repair. A- Homologous recombination and B- Nonhomologous end-joining (from [104]).

### 1.3.2.5. Mismatch Repair (MMR)

Mismatch repair pathway is involved in the repair of specific types of errors that occur during new DNA synthesis. The loss of this system accelerates the accumulation of potential mutations and predisposes to certain types of hereditary and sporadic cancers [115].

MMR system is responsible for removal base mismatches caused by spontaneous and induced base deamination, oxidation, methylation and replication errors and is also involved in the repair of DNA lesions induced by a variety of cytotoxic agents [107, 109].

The steps (presented in Figure 1.6) by which MMR proceeds are: (a) recognition of DNA lesions, (b) recruitment of additional MMR factors, (c) search for a signal that

identifies the wrong (newly synthesized) strand followed by degradation past the mismatch and (d) resynthesis of the excited tract [106, 115].

The recognition of mismatches or chemically modified bases is performed by a complex composed by the homologous proteins MSH2 and MSH6 called MutSα complex that is able to recognize base/base mismatches and short insertion/deletion loops. MSH2 can also form another complex with the mismatch repair protein MSH3, that is designated MutSβ complex that is only capable of biding to larger insertion/deletion loops [109, 115]. Upon biding to the mismatch, MutSα associates with MutLα, a complex that consist of the MutL homologous mismatch repair proteins MLH1 and PMS2. After that the excision of DNA strand containing the mispaired base is performed by exonuclease I and the new synthesis by DNA polymerase δ (polδ) [109].

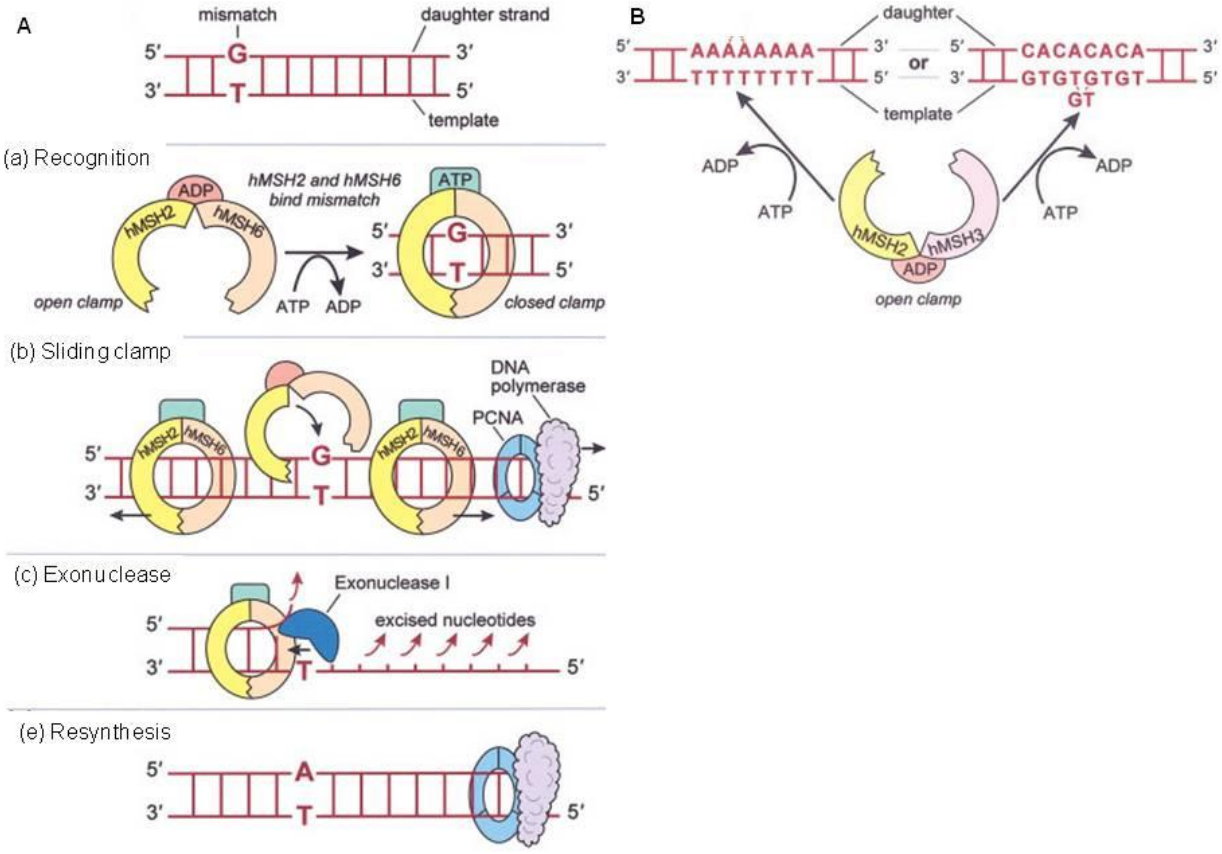



**Fig. 1.6** – Mismatch repair pathway. A- repair of a single nucleotide mismatch in S phase and B- repair of insertion/deletion errors at microsatellites sequences (from [115]).

Defects in MMR can lead to microsatellite instability, consequently heterozygotic defects in *MLH1*, *MSH2* or *PMS2* genes predispose humans to hereditary nonpolyposis colorectal cancer [110].

## 1.4. Reactive Oxygen Species

### 1.4.1. Principles

Oxidative stress by definition is an imbalance between oxidants and antioxidants, potentially leading to damage [116-118]. Oxidants are produced by an increased generation of oxygen free radicals, i.e. species containing one or more unpaired electrons, and non-radical derivatives [119]. ROS as well as reactive nitrogen species (RNS) overcomes cellular antioxidants, producing a disturbance in the equilibrium status of pro-oxidant/antioxidant reaction in living organism [117, 119].

Oxidative stress has been implicated in a various pathological processes, including cancer, inflammatory disorders, cardiovascular disease, pulmonary disease and neurodegenerative disease, as well as in ageing process [117, 120-122]. These major chronic diseases increase rapidly in both incidence and mortality as a function of age [122].

In respect to cancer, is important to note that ROS are tumorigenic due to their ability to increase cell proliferation, migration and survival, and by inducing DNA damage, all contributing to tumor initiation, promotion and metastasis [122, 123].

ROS and RNS are produced during normal cellular function, as a consequence of endogenous sources as aerobic respiration and substrate oxidation [117, 119, 122]. Besides the endogenous sources of reactive species (RS) there are several external agents that can trigger RS production. This include different types of radiation, such as ionizing and UV radiations, alcohol, tobacco smoking, diet and some environmental carcinogens, as well as viral infections [122, 124]. Figure 1.7 represents the sources and cellular responses to reactive oxygen species [125].

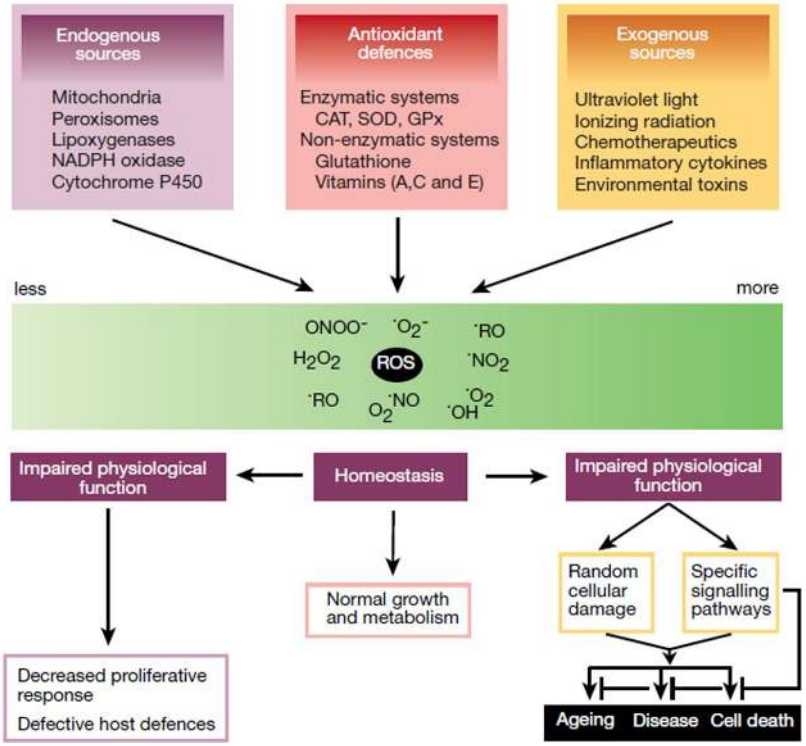



**Fig. 1.7-** The sources and cellular responses to reactive oxygen species (from [125]).

ROS include superoxide anion ($O_2^{\cdot-}$), hydroxyl radicals ($HO^{\cdot}$), hydrogen peroxide ($H_2O_2$), singlet oxygen ($^1O_2$), alkoxyl radicals ($RO^{\cdot}$), and peroxyl radicals ($ROO^{\cdot}$) [119, 126]. RNS include nitric oxide ($NO^{\cdot}$) and peroxynitrite anion ($ONOO^-$) [119, 126]. Some ROS, especially $O_2^{\cdot-}$ and $HO^{\cdot}$ are extremely unstable and reactive. On the other hand, other ROS like $H_2O_2$ or $ROO^{\cdot}$ are relatively stable, with half-lives in the range of seconds. These species may diffuse away from their site of generation, transporting the radical or the oxidant function to other target sites [116, 125]. Superoxide anion and $H_2O_2$ are formed in biological system by the partial reduction of molecular oxygen. $O_2^{\cdot-}$ is produced from one electron reduction of molecular oxygen and $H_2O_2$ is produced from a reduction of $O_2^{\cdot-}$ with a second electron or by two electron reduction of molecular oxygen. Formation of hydroxyl radical ($HO^{\cdot}$) is thought to occur through the one electron reduction of $H_2O_2$, a reaction that is facilitated by transitions metals (e.g. Cu(I) and Fe(II)), that are in reduced valence state, this is called Fenton reaction. Alternatively, hydrogen peroxide may be converted into water by the enzymes catalase or glutathione peroxidase [116, 121, 127]. Four electrons and two protons are

required to reduce molecular oxygen to water. Additionally, ROS can react with other molecules (e.g. polyunsaturated lipids, thiols and nitric oxide) producing other reactive species, such as $ROO^{\cdot}$ [116, 121].

ROS are transient due to their high chemical reactivity that leads to lipid peroxidation and oxidation of DNA and proteins [119]. The formation of 8-hydroxyguanine is a common DNA lesion, and this is one of the most extensively studied DNA lesions in humans [52]. Upon oxidation of guanine, a hydroxyl group is added to the $8^{th}$ position of the molecule and the modified product 8-hydrox-2'-deoxyguanosine (8-OHdG) is one of the predominant forms of free radical induced lesions of DNA. The presence of 8-OHdG reveals a lower fidelity in the replication process and enhances the probability of adenine incorporation into the complementary strand, giving rise to G-T transversions [49, 52]. Agents that increase levels of 8-OHdG should thus increase the risk of cancer development [52].

The oxidative DNA damage leads to alterations in purine and pyrimidine bases and deoxyribose sugar as well as cleaving the phosphodiester DNA backbone to create DNA strand breaks [122].

The production of ROS that are generated as a result of normal intracellular metabolism occurs mostly within the mitochondria of the cell [117, 125]. Low levels of ROS are beneficial and even indispensable in many biochemical processes, including in cellular response to noxia (e.g. defense against infectious agents and in function of signaling systems). However, the excess of ROS can cause severe metabolic malfunctions and cellular damage in lipids and membranes, proteins and DNA, inhibiting their normal function [117, 120, 123].

### 1.4.2. Antioxidant defenses

Antioxidants are substances that prevent or delay oxidation of cellular oxidizable substrates and involve several strategies, both enzymatic and non-enzymatic [116, 119]. Antioxidants exert their effect by scavenging reactive species or by activating detoxifying/defensive proteins [119]. Under normal conditions, antioxidant systems of the cell minimize the perturbations caused by ROS [119]. Moreover, several biological

compounds have been reported to have antioxidant functions. Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). Non-enzymatic antioxidants are represented by vitamin C (ascorbic acid), vitamin E (α-tocopherol), pyruvate, glutathione (GSH), carotenoids, flavonoids among other [117, 119, 122, 127].

## 1.5. Acrylamide

### 1.5.1. Acrylamide: production, uses and sources of exposure

Acrylamide ($CH_2$=CHCO$NH_2$) is an important industrial monomer produced by hydration of acrylonitrile with commercial availability since the mid-1950s. It is mainly used to produce water-soluble polyacrilamides, used as flocculents for clarifying drinking-water, for treating municipal and industrial waste waters and as flow control agents in oil-well operations. It was also used in soil stabilization and in grouting for repairing sewers and manhole. The monomer itself is also handled in many molecular biology and genetic engineering laboratories for the preparation of electrophoresis gels [54, 128-130]. In the workplace the major routes of exposure appear to be dermal absorption of acrylamide monomer from solution and inhalation of dry monomer or aerosols of acrylamide solution. Additionally, AA has other uses such as cosmetic additives (e.g. creams, body lotions, shampoos) and is also a component of tobacco smoke (1-2 μg/cigarette) [54].

Besides the industrial and laboratory uses the general population is exposed to varying amounts of AA via diet. In fact, recently, it was discovered that AA can be formed in significant amounts and measured at significant concentrations in many common human foods during high-temperature frying, roasting or baking. However this compound is not typically found in boiled or micro-waved food [128, 129, 131, 132]. Moreover, the general population is exposed to small quantities of AA in drinking water, refined with poliacrylamide [133].

Acrylamide can be generated from food components during heat treatment as a result of the Maillard reaction between an amino acid, primarily asparagine (the major amino acid in potatoes and cereals) and reducing sugars, such as glucose [134, 135]. AA formation begins at temperatures between 160 and 180ºC [132] and the products of Maillard reaction are responsible for much of the flavor and color generated during baking and roasting [129, 134, 135]. Figure 1.8 represents the proposed pathway for the formation of acrylamide by Heatox 2007 [136].

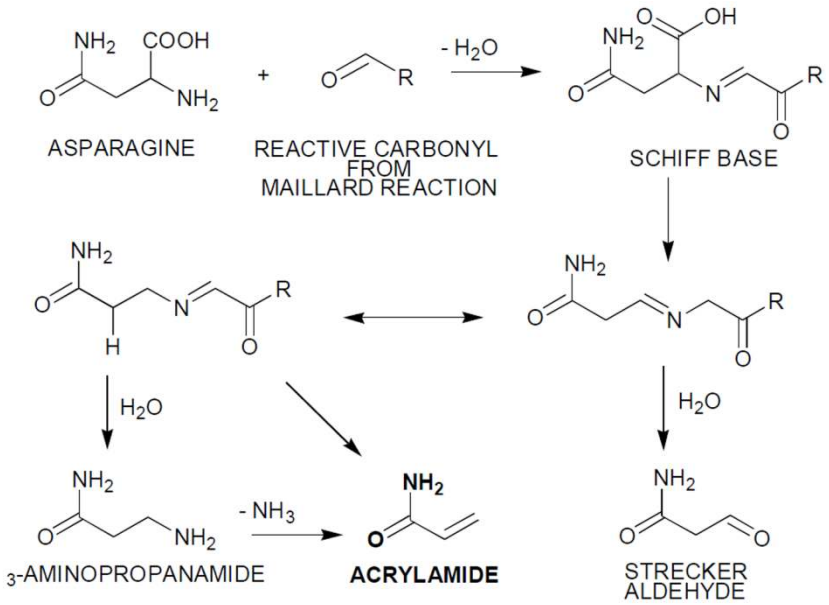



**Fig. 1.8-** Proposed pathway for the formation of AA after thermal processing (from [136]).

The proposed mechanism for AA formation (see Figure 1.8) involves the formation of a Schiff base from the reaction of a carbonyl compound with asparagine. Decarboxylation of the Schiff base, in a Strecker-type reaction, gives an unstable intermediate that can hydrolyze to 3-aminopropamide, which on elimination of ammonia yields AA. Alternatively, the decarboxylated Schiff base could form AA via elimination of an imine [136]. In addition to the Maillard reaction, alternative routes for the formation of AA have been proposed [137].

AA is very soluble in water, to the extent of 215.5 g/100ml at 30ºC [131], but is equally well soluble in some organic solvents including methanol and ethanol [138].

Acrylamide was evaluated by the International Agency for Research on Cancer in 1994 as "probably carcinogenic to humans" (IARC, Group 2A) on the basis of the positive bioassay results in mice and rats, supported by evidence that AA is biotransformed in mammalian tissues to a chemically reactive genotoxic metabolite named glycidamide (GA). In the European Union classification system it is classified as a category 2 carcinogen, a category 2 mutagen and as toxic to reproduction in category 3 [54, 130, 131, 133].

### 1.5.2. Acrylamide dietary exposure

More than one-third of the calories we consume in each day come from foods with detectable levels of AA [132]. Average daily intake was estimated to be ranged from 0.3 to 2.0 μg/kg bw in the general population. For high percentiles consumers (90th to 97.5th) intake estimates ranged from 0.6 to 3.5 μg/kg bw per day, and up to 5.1 μg/kg bw per day for the 99th percentile consumer [139]. Children would generally have exposures 2-3 times higher and therefore they are at higher risk than adults [54, 140]. This may be due to a combination of children's higher caloric intake relative to body weight as well as their higher consumption of certain AA-rich foods, such as French fries and potato crisps, but butter biscuits and sweet biscuits are also important sources. [132], these products contribute to approximately 55-65% of the total mean intake [141].

Studies conducted in Sweden in 2002 showed that high levels of AA were formed during the frying or baking of a variety of foods and this finding was quickly confirmed by authorities in Europe and in United States [142]. Due to the high concerns about the possible public health risks from dietary exposure to AA several reports were performed with the purpose to reduce dietary AA intake. This requires that dietary levels of AA should be further reduced by appropriate technology in food processing and that AA levels should be monitored regularly in commercially distributed food items. Moreover, the consumers must be instructed and follow appropriate rules of food preparation, especially for home-made food [129].

In 2005 and according to Joint FAO/WHO Expert Committee on Food Additives (JECFA) a report showed that the major contributing foods to total exposure to AA, for most countries were potato chips (French fries) (16-30%), potato crisps (Chips) (6-46%), coffee (13-39%), pastry and sweet biscuits (Cookies) (10-20%) and bread and rolls/toasts (10-30%). Conversely others foods items contributed less than 10% of the total exposure. Given that, it can be said that foods rich in AA precursors are largely derived from vegetable sources, such as potatoes and cereals, but apparently not from animal sources [139, 143].

The more recent results on AA levels in certain foodstuffs were reported by the European Food Safety Authority (EFSA) in 2010. This report describes the results of the monitoring exercise in the period from 2007 to 2009 from a total of 23 Member States, plus Norway submitted 10366 results to EFSA for the three-year period [144]. Portugal was one of the countries that did not provide results of AA levels in food items, therefore not contributing for this EFSA report. However, a study is currently being performed in young adult Portuguese population and the overall results obtained in this study showed that the estimation of the amount of AA consumed in the large group of participants was approximately 0.35, 0.56, 0.87, 1.35 and 1.74 μg/kg wt/ day for 25, 50, 75, 90 e 95 percentiles respectively.

The 3728 results from 2008 were compared with the 3281 results collected in 2007 (Table I.2). The product categories potato crisps, instant coffee and substitute coffee showed statistically significantly higher levels of AA in 2008 compared to 2007. On the other hand, French fries and fried potato products for home cooking, soft bread, bread not specified, infant biscuit, biscuit not specified, muesli and porridge and other products not specified showed statistically significantly lower levels of AA in 2008 compared to 2007. There were no statistically significant differences in AA level for the other food groups [144]. This report suggests lower AA values in 2008 compared to 2007.

**Table I.2 -** Acrylamide levels in various food reported by the Member States of European Union and Norway in 2007 and 2008 (Adapted from [144]).

| Food | 2007 | | 2008 | |
|---|---|---|---|---|
| | Median (µg/kg) | Maximum (µg/kg) | Median (µg/kg) | Maximum (µg/kg) |
| **Biscuits** | | | | |
| Crackers | 195 | 1526 | 185 | 1042 |
| Infant | 100 | 2300 | 64 | 1200 |
| Not specified | 173 | 4200 | 126 | 1940 |
| Wafers | 118 | 1378 | 109 | 2553 |
| **Bread** | | | | |
| Bread crisp | 116 | 2430 | 107 | 1538 |
| Bread soft | 30 | 910 | 30 | 528 |
| Non specified | 58 | 2565 | 19 | 86 |
| **Breakfast cereals** | 100 | 1600 | 75 | 2072 |
| **Cereal-based baby food** | 38 | 353 | 25 | 660 |
| **Coffee** | | | | |
| Instant | 188 | 1047 | 482 | 1373 |
| Not specified | 183 | 1158 | 210 | 734 |
| Roasted | 197 | 958 | 164 | 1524 |
| **French fries** | 246 | 2668 | 220 | 2466 |
| **Jarred baby food** | 30 | 162 | 25 | 297 |
| **Other products** | | | | |
| Gingerbread | 226 | 3615 | 227 | 3307 |
| Muesli and porridge | 156 | 805 | 30 | 112 |
| Not specified | 134 | 2529 | 60 | 2592 |
| Substitute coffee | 334 | 4700 | 702 | 7095 |
| **Potato crisps** | 413 | 4180 | 436 | 4382 |
| **Home-cooked potato products** | | | | |
| Deep fried | 182 | 1661 | 152 | 1220 |
| Not specified | 150 | 2175 | 75 | 3025 |
| Oven fried | 260 | 941 | 172 | 1439 |

*Note*: Median values are upper bound values.

According to this EFSA report, the area of potato products has drawn much attention because of their important contribution to the AA exposure based both on a high consumption of the products and on a relatively high content of AA. Potato crisps were identified as a food product with potential for high levels of AA formation [145]. Given their popularity as a snack food, particularly among younger age groups, it is important to reduce its AA content [144].

Moreover, there are a second large group of products contributing to AA exposure which are cereals. From the comparison of AA data between 2007 and 2008 it was observed that the overall trend in cereal products tends towards lower AA content showing a statistically significant decrease in soft bread, infant biscuit and muesli and porridge. However, no statistical difference in AA content between 2007 and 2008 was found for the other cereal products [144].

On the other hand, coffee is also an important contributor to AA exposure. The results of laboratory scale experiments have led to the conclusion that only limited process options are available to reduce AA levels without affecting the quality in respect to the consumer acceptance of a product. Other conclusion in studies at pilot scale showed that asparagine content of dried chicory was correlated to the formation of AA [144].

The wide variations in levels of AA in different food categories as well as in different brands of the same food category (e.g. French fries, potato chips) appears to result from the variation in processing conditions (temperature; time; nature of frying oil; nature of food matrix) [128, 129]. Large variations are also to be expected during home cooking, although this aspect has been less well documented. Additionally, the composition of the food also has an influence, crucially the content of free asparagine and reducing sugars. Storage and seasonal variations can also occur and other important factors could be pH and water content [139].

In order to confirm whether the AA levels tend indeed towards a decrease over time more food samples need to be collected and analyzed in coming years [144].

### 1.5.3. Acrylamide toxicokinetics

Acrylamide is reactive in three different ways. First, it can undergo radical-mediated polymerization. Secondly, AA has an alpha, beta unsaturated double bond, that reacts with nucleophile, including amino and thiol groups in amino acids and proteins by Michael additions. This alpha, beta double bond is responsible for much of its activity. The thiol addition represents a detoxification pathway by yielding primarily

AA-glutathione conjugates as urinary excreteable compounds. On the other hand it can also result in alkylation of proteins and can also bind to DNA in a similar "Michael type" addition, however with low reactivity [54, 131, 146]. Thirdly, AA can be metabolized to glycidamide (GA) an epoxide derivative, presumably by cytochrome P4502E1 (Fig. 1.9), being readily reactive toward DNA and other molecules [146].

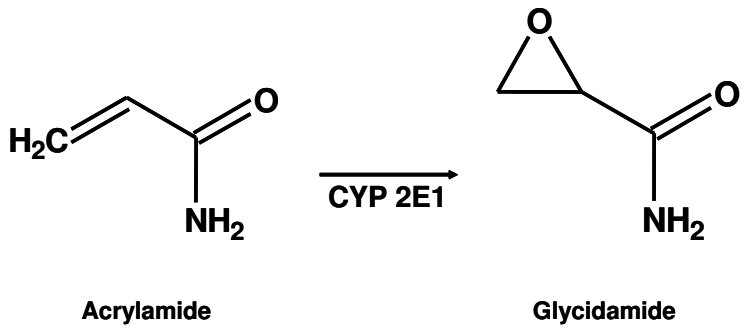



**Fig. 1.9** - CYP2E1 mediated biotransformation of AA to GA.

For several decades is known that AA is metabolized to GA, but only in 1993 Bergmark *et al* showed the first evidences for formation for GA in humans. These evidences have been shown indirectly in the hemoglobin workers exposed to relatively high levels of AA, through the detection of Hb-adducts of GA [147].

Glycidamide is an epoxide metabolite which is genotoxic in a variety of *in vitro* and *in vivo* test system [131, 138]. GA has an epoxide group that appears generally more reactive with hemoglobin that the double bond of AA, and GA has been reported to be 100-1000 times more reactive with DNA than AA [131]. Due to this, the genotoxic effect of AA is mostly attributed to GA, focuses much of the toxicological interest of this compound in its metabolite and making it the objective of a large number of studies. However, the role of GA in the toxicity of AA is not fully understood and continues to be worthy of attention by the scientific community.

After oral administration, AA is rapidly absorbed by the gastrointestinal tract in all species, including rats, mice and human. On the other hand, dermic absorption and inhalation is much less efficient [148, 149]. AA passes in the blood and is widely distributed in the body, including breast tissue, due to hydrosolubility, in all animal

species so far investigated (rats, mice, dogs and mini-pigs) [54, 138, 150, 151]. Additionally, it has been shown that AA could cross the blood/placenta barrier in a human placenta *in vitro* model as well as the blood/breast milk barrier *in* vivo of lactating mothers [152].

Ingested AA is taken up into circulation and metabolism of AA can proceed according to Figure 1.10. As already previously seen, AA is largely oxidized in mice, rats and humans to GA, being cytochrome P450 2E1 the more plausible candidate for its oxidation. This enzyme also oxidizes alcohol and is induced by ethanol [54, 153-156]. Secondly both AA and GA can be conjugated with GSH yielding excreatable mercapturic acids conjugates, which can be eliminated via the urine [86]. The conjugation of AA with GSH yield N-acetyl-S-(3-amino-3- oxopropyl)cysteine and when GA reacts with GSH yield N-acetyl-S-(3-amino-2-hydroxy-3-oxopropyl)cysteine and N-acetyl-S-(carbamoyl-2-hydroxyethyl)cysteine [54, 129, 153]. Thirdly GA hydrolysis can occur via epoxide hydrolase (EH) forming 2,3-dihydroxypropionamide [153, 155].

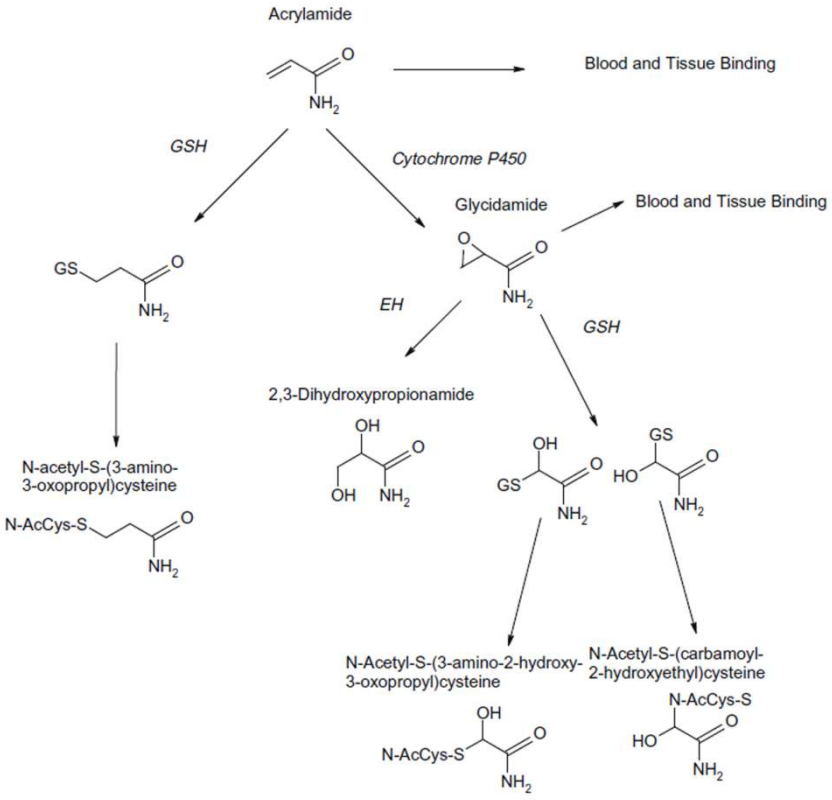



**Fig 1.10** - Proposed mammalian metabolic pathway for AA. GSH, glutathione; EH, epoxide hydrolase (from [153]).

Except for the differences related to the metabolism of AA and its metabolites, few quantitative differences are expected between rats and humans with respect to absorption, distribution and excretion, in part due to the high hydrosolubility [153]. In what concerns the expected differences between humans and rats it is important to note that human metabolizes much less AA to GA compared to rats in similar doses, and the GA produced in rats is conjugated by GSH to a much larger extent than in humans. This is very important with respect to risk assessment [129, 153]. Humans, on the other hand, detoxify and eliminate GA via hydrolysis almost exclusively, while rats only hydrolyze GA to a limited extent. In view of this, humans will have a much lower blood level of GA than rats, for a given dose of AA [153]. Presently, one can say that in humans, at relatively low doses GA is formed at higher extent than in rats, most likely because of the higher levels of CYP2E1 [54].

Several studies on the toxicokinetics of AA were carried out [148, 149, 153, 156]. The overall results suggest conjugation of AA with GSH exceeds the formation of reactive metabolite GA, having a bioavailability of AA in humans of about 20-49%. It is metabolized to GA, than for your turn is detoxified by conjugation with GSH [148].

Both AA and GA are equally distributed among the tissues and have half-lives of about 5 h in rats. The conversion of AA to GA is saturable, ranging from 50% of very low doses to 13 % at 100 mg/Kg bw in rats [54].

It is also important to note that there are several exogenous factors that are very important in the formation of GA in humans that should be taken into account, such as alcohol consumption, smoking habits, age and sex [157, 158]. Two studies by Vikström *et al* [158] and Vesper *et al* [157] investigated whether alcohol (ethanol) consumption might have an influence on the metabolism of AA to GA in humans exposed to AA through foods. In these studies a decrease of GA adducts associated with alcohol consumption was observed. This can be explained due to a competitive effect between ethanol and AA as both are substrates for cytochrome P4502E1. These results strongly indicate that ethanol influence metabolism of AA to GA.

The thorough knowledge of AA metabolism is very important, since only the knowledge of these mechanisms will allow us to dissipate any doubts regarding the toxicology of this compound.

### 1.5.4. Adducts of AA and GA

#### 1.5.4.1. Hemoglobin adducts

An important reaction of AA with protein is the formation of hemoglobin (Hb) adducts. These adducts are formed at the site of sulfhydryl (SH) groups and on the amino groups of the N-terminal aminoacids ($\alpha$-$NH_2$), and have been widely used to estimate internal exposures in human biomonitoring studies [54, 138]. Hemoglobin adducts from direct reaction of AA and from reaction with GA have been detected in rodents administered AA, in exposed humans, and in cigarette smokers [141]. Data showed that Hb adduct formation was linear in a dose response manner when the epoxide GA was administrated in experimental animals [138]. Moreover, preliminary studies that measured concentrations of AA- and GA-haemoglobin adducts in rodents and humans with background exposure to AA through the diet suggested that there may be species differences in the relative formation of GA with mouse > rat >human. However, the formation of GA and AA adducts with Hb is directly proportional in man and rat [54]. Additionally, the detection of Hb-adducts for GA in both rodents and humans confirms the formation of GA *in vivo* in humans [54].

Smoking is one example of an exogenous factor that has been studied by correlating exposure of tobacco smoking and Hb-AA and Hb-GA adducts. These studies revealed levels of hemoglobin adducts significantly higher in smokers than in non-smokers [159, 160].

Hb-adducts are not used as an indicator of toxicity. However, they are used as a measure of human exposure to electrophilic compounds, for example as a marker of *in vivo* exposure to AA [54]. Some studies have used hemoglobin adducts of AA and GA as biomarkers of AA exposure to determine the internal exposure dose [147, 159, 161, 162]. Hemoglobin adducts were also correlated with neurotoxicity, but there has been no systematic standardization of hemoglobin adducts with dose [163].

### 1.5.4.2. DNA adducts

Acrylamide adduct formation with DNA has also been reported, although the reaction is very slow. AA itself is of low DNA reactivity under *in vitro* conditions but after metabolic activation specific DNA adducts on the basis of GA were observed [164]. Due to this, the genotoxicity of AA has been mainly attributed to the epoxide metabolite GA [146].

AA is proposed to interact with DNA, giving rise to promutagenic AA DNA-adducts. The structural analysis of reaction products of AA with DNA *in vitro* revealed the formation of the following adducts in descending order of abundance, N1-(2-carboxyethyl)-adenine, N3-(2-carboxyethyl)-cytosine, N7-(carbamoylethyl)-guanine, N6-(2-carboxyethyl)-adenine and N1-(carboxyethyl)-guanine (Figure 1.11) [146, 165].

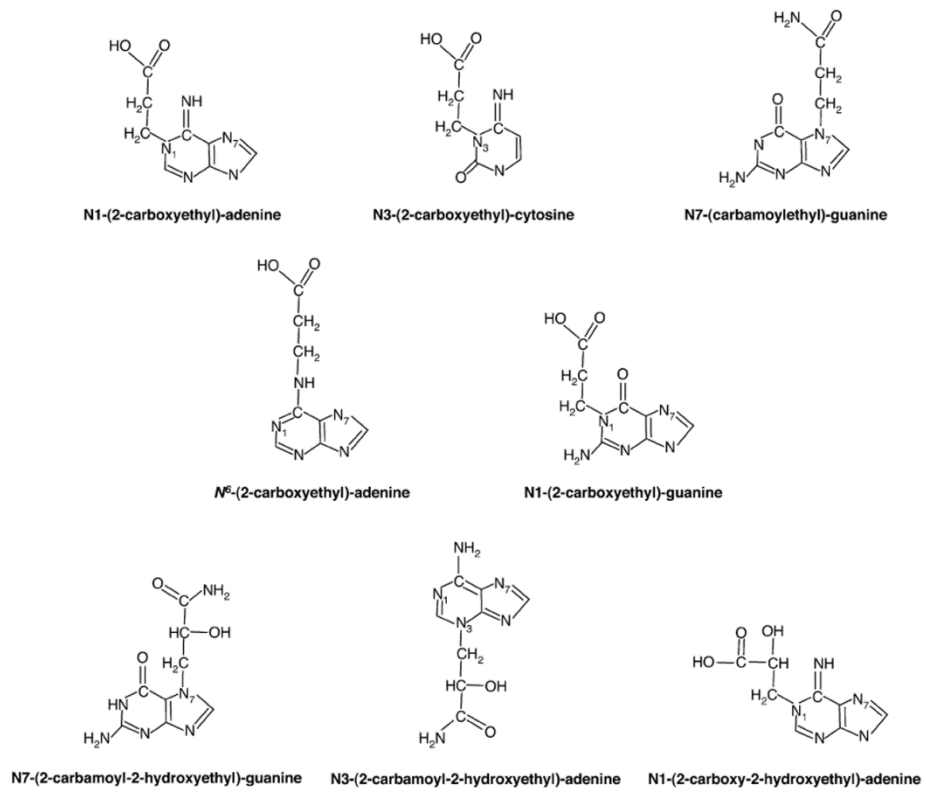



**Fig. 1.11** - Chemical structures of major DNA adducts of AA and GA (from [146]).

On the other hand GA forms adducts with DNA *in vivo* and *in vitro* in considerable amounts [164]. The predominant adduct detected in mice and rats has been a GA-guanine adduct the N-7-(2carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua),

formed by reaction of the DNA with the epoxide metabolite GA. N3-(2-carbamoyl-2-hydroxyethyl)adenine (N3-GA-Ade) and N1-(2-carboxy-2-hydroxyiethyl)-2'-adenine (N1-GA-Ade) were also identified as additional GA-derived DNA adducts, (Figure 1.11) [164, 166]. Both N7-GA-Gua and N3-GA-Ade are promutagenic, because they can undergo spontaneous depurination, producing an apurinic (AP) sites [146, 167]. It has been suggested that the abasic sites that are produced by depurination of N7-GA-Gua are likely to promote incorporation of deoxyadenosine during DNA replication leading to G-T transversions [146, 167]. The N3-GA-Ade also can block transcription, which can lead to sister chromatid exchange, S-phase arrest, chromosomal aberration and cytotoxicity [164]. N1-GA-Ade is also highly promutagenic, because of its impaired base-pairing potential [146, 168].

DNA adducts have been found in liver, lung, testis, leukocytes, and kidney of mice and in liver, thyroid, testis, mammary gland, bone marrow, leukocytes, and brain of rats treated with either AA or GA [139]. These adducts, formed in rodents, showed the formation of higher amounts of DNA adducts by treatment with GA than after AA treatment, at all doses tested [131, 146]. DNA adducts formation from AA was saturable, while formation of most DNA adducts from GA was dose-dependent at the doses tested [131, 146].

In mice higher levels of DNA adducts were detected when compared to rats. This is already expected due the correlation with the greater metabolic conversion of AA to GA in mice compared to rats [54, 138]. According to Maniére *et al* [51] the N7-GA-Gua was detected in similar levels in brain and liver and at lower levels in testes of rats treated with a single oral dose of AA. The overall DNA adducts in adult mice treated with GA were 1.2-1.5 fold higher than those in their AA treated counterparts [146]. DNA adducts formation after AA exposure in humans is still lacking [54, 138].

### 1.5.5. Genotoxicity of acrylamide and glycidamide

The genotoxicity of AA as well as of its reactive metabolite epoxide GA, has been extensively studied. AA is both clastogenic and mutagenic in mammalian cells *in*

*vitro* and *in vivo*. In addition, dominant lethality studies have showed that AA is a germ cell mutagen in male rodents. Additionally, the mutational spectra produced by AA and GA in transgenic mouse cells are consistent with formation of promutagenic purine DNA adducts *in vivo* [131]. Furthermore, there is some evidence of the involvement of free radicals in AA genotoxicity, leading to oxidative modification of pyrimidines [169].

#### 1.5.5.1. Genotoxicity in Prokaryotes

AA was not mutagenic in bacterial systems, since did not induce gene mutations in different strains of *Salmonella typhimurium* (Ames test), in the presence or in the absence of an exogenous activating system. In contrast, GA was mutagenic in bacterial system, since it induces gene mutations in *S. typhimurium* strains TA1535 and TA100 with and without metabolic activation [54, 129, 138]. The fact of AA did not induce gene mutations may be related with the scarce presence or lack in the S9 mix of the specific isozyme (as been already seen the most plausible is P4502E1) capable of metabolizing small molecules [54]. Moreover, a study *in vitro* demonstrated that AA is not activated in presence of the most common exogenous system of metabolic activation (rat liver S9 mix) [170].

#### 1.5.5.2. Genotoxicity in mammalian cells

*In vitro,* AA generated positive results for mutagenicity even without metabolic activation. These positive results showed mutations thymidine kinase *(TK)* locus in mouse lymphoma cells [171] and in human lymphoblastoid TK6 cells [170] but only for high concentrations (>10 mM). However, in V79 cells AA was inactive up to concentration of 10 mM in hypoxanthine-guanine phosphoribosyl transferase *(Hprt)* mutagenicity test [172]. On the other hand, GA was mutagenic in relatively low concentrations in both *TK* and *Hprt* assays [170-172]. Furthermore, molecular analysis of the TK mutants revealed that AA predominantly induced loss of heterozygosity mutation like spontaneous one while GA-induced primarily point mutations [170].

*In vivo,* both AA and GA originated dominant lethal mutations in rodents [173]. In Big Blue rats, both compounds significantly increased lymphocyte *Hprt* mutant

frequency [174, 175]. However, neither compound increased the *cII* mutants in testis, mammary gland and liver, while both compounds induced weak positive increases in bone marrow and thyroid [174]. Opposing results were observed by Majanatha *et al* in relation to *cII* mutants frequencies that were increased in liver for high doses of AA and GA. Moreover, molecular analysis of the mutants indicated that AA and GA produced similar mutations spectra, of which, the predominant were G to C tranversions and frameshifts [175].

Previously in another *in vitro* study, GA showed to be more mutagenic than AA at any given dose. The spectrum of GA-induced cII mutations was statistically significant different from the spectrum of spontaneously occurring mutations in control-treated cells. Further cells treated with GA or AA had more A→G transitions and G→C tranversions and moreover cells treated with GA had more G→T transversions [53]. This last mutation is compatible with *in vivo* studies and the authors concluded that although both AA and GA are mutagenic in mice, the mutagenicity of AA is based on the capacity of its epoxide metabolite GA to form DNA adducts [53, 175], pointing out that the mutation spectrum been totally compatible with adducts of adenine and guanine previously identified [164].

Several studies showed pronounced evidence about the role of GA mutagenicity *in vivo* and *in vitro*. On the contrary, in relation to AA only a weak *in vitro* mutagenic potential was attributed, showed possible clastogenic effects. AA is clearly a direct-acting clastogen in mammalian cells in which it also induces, at lower extent, aneuploidy, polyploidy and other mitotic disturbances [54]. In fact the clastogenicity of AA have been reported in several studies, namely cytogenetic studies [176-178].

The *in vivo* exposure to AA induced positive results in chromosomal alterations in mouse bone marrow cells but negative in spermatoigonia [179].

On the other hand, *in vitro* studies reported that AA induced sister chromatid exchanges and chromosomal aberrations in V79 cells [176, 178]. AA also showed to be clastogenic in micronucleus assay and presented DNA strand breaks (comet assay) in HepG2 cells [180]. However, significant micronucleus inductions were only found in human blood cells [172] and TK6 cells [170]. Others authors also found DNA damage in V79 and in Caco-2 cells but for AA high concentrations (6 mM) [181].

In relation to GA a significant level of DNA damage was reported *in vitro* in testicular cells of mice and human peripheral blood lymphocytes [182]. Also the induction of micronuclei in human TK6 cells [170] and in human blood [172] has been reported. Other studies observed an increase in micronuclei in peripheral blood [175, 183, 184] and in bone marrow of mice [179] but not in rats [174, 184] when administered with AA. Paulsson *et al* (2003) showed that mice suffer a dose-dependent increase in micronucleus when exposed to GA [185]. These genotoxicity differences may be related with the different metabolism between rats and mice [186]. Overall, the studies *in vitro* of DNA damage confirmed the major mutagenic potential for GA in relation to AA, not only for the significant increases of DNA damage with low doses of compound, but also the positive results obtained in all cell lines studied [170, 181, 182].

Additionally studies with CYP2E1 in mice also suggest strong evidences about genotoxicity of AA trough GA in germ cells [187] and in somatic cells [168].

Overall in cytogenetic studies both AA and GA has demonstrated being clastogenic, however the reactive metabolite GA showed a greater potent to induce CAs, MN, SCEs and DNA strand breaks [170, 176, 188].

### 1.5.6. Animal carcinogenicity

Acrylamide is carcinogenic to experimental mice and rats, causing tumors at multiple organ sites, in both species given in drinking water or by others means. AA was tested for carcinogenicity in two experiments in Fischer 344 rats by oral administration [189, 190]. In these studies an increase of the incidence of peritesticular mesotheliomas and folicular adenomas of the thyroid was observed in males and of thyroid follicular tumors, mammary tumors, glial tumors of the central nervous system, oral cavity papillomas, uterine adenocarcinomas and clitoral gland adenomas in females [189, 190]. These positive biosassays of AA carcinogenicity in experimental animals establish that AA is a multiorgan carcinogen in both rats and mice. This is of high importance, since they indicate that AA presents a potential carcinogenic hazard to humans [131].

### 1.5.7. Epidemiologic studies

Epidemiology is the study of the distribution and determinants of disease in a population that can suggest association but not causation, between exposure to an agent and an outcome, e.g., disease [53, 132]. Several epidemiologic studies were conducted with the aim of assess the association between AA intake and the risk of cancer in many sites, however, there are some conflicting results.

In the 1980's, two studies investigating the correlation between occupational AA exposure and cancer mortality, were carried out by Sobel *et al* [191] and Collins *et al* [192]. In these studies occupational AA exposure was evaluated in two cohorts of industrial workers who were exposed in the monomer production and polymerization industries. After the analysis of cancer incidence in those workers, no consistent effect of AA exposure on cancer incidence at any site was identified [131, 138].

In 1999, an extended and updated investigation of the mortality experience of part of the cohort originally studied by Collins *et al* [192] was undertaken by March *et al* [193]. In an exploratory exposure-response analysis of rectal, oesophageal, pancreatic, and kidney cancer, increased standardized mortality ratios for some categories of exposure to AA were observed but little evidence of an exposure-relation was found. These results corroborated the original cohort study findings [193]. An updated follow up done by the same authors [194] concluded the same results, since AA exposure at the level present in the study sites was not associated with elevated cancer mortality risks.

In recent years, due to the high concern of AA exposure, several studies evaluated the carcinogenicity of AA/GA in relation to dietary intake in various European countries, including Sweden, Switzerland, Netherlands, Norway and Italy and also in American population were carried out and are summarized in Table I.3. Several sites of cancer risk were assessed and the AA intake was carried out for estimative with bases in food frequencies questionnaires (FFQs) to assess diet. It is, however, not well-known, whether FFQs can accurately measure AA [172] intake in the diet. AA content in a particular food varies with specific cooking and processing methods for both homemade and commercially prepared foods, which makes measuring individual intake difficult [195]. Only recently case-control studies have examined the relationship

between the AA-Hb and GA-Hb adducts and breast and prostate [195] cancer risk. They analyzed N-terminal hemoglobin adduct levels of AA and its genotoxic metabolite, GA in blood samples [195, 196].

As shown in Table I.3 most of the results of the case-control and cohort studies done revealed no significant correlation between frequent consumption of foods with high levels of AA and the increase of cancer risk in various sites, like bowel, bladder, kidney, prostate, among others. However, positive results have also been found, particularly in relation to breast, ovarian, endometrial, lung, renal and esophageal tissues.

**Table I.3 -** Epidemiological studies of dietary AA intake and cancer risk (adapted from [197]).

| Cancer site | Design | Population | Sample size | Cancer risk | Published |
|---|---|---|---|---|---|
| Colon, rectum,<br>Bladder<br>kidney | case-control | Swedish men and women | 591 cases; 538 controls<br>263<br>133 | -<br>-<br>- | [198] |
| Renal | case-control | Swedish men and women | 379 cases; 353 controls | - | [199] |
| Breast | cohort | Swedish women | 43404<br>667 cases | - | [200] |
| Colon, rectum | cohort | Swedish women | 504/237 cases(colon/rectal)<br>823072 person-years | - | [201] |
| Breast | Case-control | American women | 582 cases; 1569 controls | + | [202] |
| Oral/pharynx<br>Esopgagus<br>Large bowel<br>Larynx<br>Breast<br>Ovary<br>Prostate | Case-control | Italian and Swiss men and women | 749 cases; 1772 controls<br>395 cases; 1066 controls<br>2280 cases; 4765 controls<br>527 cases; 1297 controls<br>2900 cases; 3122 controls<br>1031 cases; 2411 controls<br>1294 cases; 1451 controls | -<br>-<br>-<br>-<br>-<br>-<br>- | [203] |
| Endometrial<br>Ovarian<br>Breast | Sub-cohort | Dutch women | 2589; 327 cases<br>300 cases<br>1835 cases | +<br>+<br>- | [204] |
| Breast | Case-control | Danish women | 374 cases; 374 controls | + [a] | [196] |
| Renal cells<br>Bladder<br>Prostate | Sub-cohort | Dutch men and women | 5000; 339 cases<br>1210 cases<br>2246 cases | +<br>-<br>- | [205] |
| Lung | cohort | Dutch men and women | 58279 men; 62573 women<br>2649 cases | - | [206] |
| Brain | cohort | Dutch men and women | 5000; 216 | - | [207] |
| Breast | cohort | Swedish women | 61433; 2952 cases | - | [208] |
| Ovarian | cohort | Swedish women | 61057; 368 cases | - | [209] |
| Endometrial | cohort | Swedish women | 61226; 687 cases | - | [210] |
| Colorectal | cohort | Swedish men | 45306; 676 cases | - | [211] |
| Prostate | cohort | Swedish men | 45306; 2696 cases | - | [212] |
| Oral cavity<br>Oro-hypopharynx<br>Larynx<br>Thyroid | cohort | Dutch men and women | 120852; 101 cases<br>83 cases<br>180 cases<br>66 cases | + [b]<br>-<br>-<br>- | [213] |
| Prostate | Case-control | Swedish men | 1499 cases; 1118 controls<br>170 cases; 161 controls | -(FFQ)<br>-(AA-Hb) | [195] |
| Breast | cohort | American women | 90628; 1179 cases | - | [214] |
| Breast | cohort | English women | 33731; 1084 cases | + [c] | [215] |
| Breast | cohort | Dutch women | 62573; 2225 cases | + [d] | [216] |
| Endometrial<br>Ovarian<br>Breast | cohort | American women | 69019; 484 cases<br>80011; 416 cases<br>88672; 6301 cases | +<br>+<br>- | [217] |
| Lung<br>Prostate<br>Urotherial<br>Colorectal<br>Stomach<br>Pancreatic<br>Renal<br>Lymphomas | cohort | Finnish men | 27111; 1703 cases<br>799 cases<br>365 cases<br>316 cases<br>224 cases<br>192 cases<br>184 cases<br>175 cases | +<br>-<br>-<br>-<br>-<br>-<br>-<br>- | [218] |

**Table I.3 (continued)**

| Cancer site | Design | Population | Sample size | Cancer risk | Published |
|---|---|---|---|---|---|
| Esophageal | Case-control | Swedish men and women | 618 cases; 820 controls | +[e] | [219] |
| Prostate | cohort | American men | 47896; 5025 cases | - | [220] |

(+) significant association between AA intake and cancer risk; (-) absence of a positive association.

[a] Association with hormonal receptor status; [b] positive association for female non-smokers; [c] weak association with premenopausal breast cancer; [d] association with hormonal receptor status in postmenopausal never smoking women; [e] stronger association among overweight or obese persons was indicated.

In 2007, Hogervorst *et al* [204] investigate the association between AA intake and endometrial, ovarian and breast cancer risk. In this study the risk of breast cancer was not associated with AA intake. However, an increased risk of postmenopausal endometrial and ovarian cancer with increasing dietary AA intake was observed [204]. The same authors examined the association between AA intake and renal cell, bladder and prostate cancer and found some associations between dietary AA intake and renal cell cancer risk. However, it was a slight association that was noted only after the authors attempted to adjust for smoking, hypertension, body mass index, and fruit and vegetable consumption [205]. Negative results were also obtained by other authors in relation to prostate and breast cancer [195, 217]. On the other hand, there are other studies that correlate positively the dietary intake of AA and breast cancer risk. For example, Michels *et al* (2006), correlate diet during preschool age and risk of breast cancer and observed a possible association between diet before puberty and the subsequent risk of this type of cancer [202]. Furthermore, Olesen *et al* [196] used AA-Hb and GA-Hb adducts as biomarkers of acrylamide exposure founded a weak association between GA hemoglobin levels and incidence of estrogen receptor positive breast cancer after adjustment for smoker behavior. This was the first epidemiologic study done using AA-Hb and GA-Hb adducts as biomarkers. Later Wilson *et al* [195] using the same type of biomarkers (AA-Hb) found no association between AA-Hb adducts with risk of prostate cancer. Another study done by Pedersen *et al* [216] also showed some indications of a positive association between dietary acrylamide intake and receptor positive of breast cancer risk in postmenopausal never smoking women. Burley *et al* [215] showed a weak association of premenopausal breast cancer, however requires further investigation. Lastly Shouten *et al* [213] founded some association

between dietary AA intake and oral cavity cancer risk for female nonsmokers and Lin *et al* [219] found that dietary intake of AA might be a risk exposure for esophageal cancer, mainly among overweight or obese persons. Furthermore, dietary AA intake was positively associated with the risk of lung cancer by Hirvonen *et al* [218] in a Finnish male smoker's population.

The results obtained concerning animal and human data on AA risk diverge. This should be analyzed in light of the strengths and limitations of epidemiologic studies. One possible explanation for this disparity is that the adverse effects of AA are present in the cohort studies, but cannot be observed in the study populations due to limitations in statistical power to detect pathological events. Alternatively, the disparity may be due to invalid assumptions made in risk assessments [132]. Two common assumptions in human health risk assessment include (1) extrapolation in a systematic manner to predict response rates in humans according to tumor or other pathology response rates in a test species and (2) tumor and other pathology response rates observed following high dose exposures can be extrapolate to predict response rates following low-dose exposure [153]. It is important to note that the level of conversion of AA to GA may be different at very low versus high doses, and cellular protective mechanisms such as DNA repair may effectively lower the deleterious effects of AA and GA at the lower doses. AA exposure may also produce different effects when delivered with a multitude of nutrients and other compounds in foods rather than as a single additive in water, since the bioavailability of AA is also different according to the source of AA exposure [132, 153]. In addition, the tumors observed in rodents and the increased risk observed in some studies suggests that AA can influence the hormonal systems, for which rodents could not be a good model [221].

## 1.6. References:


1. Sutandyo N: **Nutritional carcinogenesis**. *Acta MedIndones* 2010, **42**(1):36-42.

2. WHO: **Global cancer rates could increase by 50% to 15 million by 2020**. *[serial online]* 2003.

3. World Cancer Research Found / American Institute for Cancer R: **Food, Nutrition, Physical Activity and the Prevention of Cancer: a Global Perspective**. In., edn.; 2007.

4. Doll R, Peto R: **The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today**. *JNatlCancer Inst* 1981, **66**(6):1191-1308.

5. Manson MM, Benford DJ: **Factors influencing the carcinogenicity of food chemicals**. *Toxicology* 1999, **134**(2-3):93-108.

6. Gibson TM, Ferrucci LM, Tangrea JA, Schatzkin A: **Epidemiological and clinical studies of nutrition**. *SeminOncol* 2010, **37**(3):282-296.

7. Leonard A: **Les mutagènes de l'environment et leurs effects biologiques.** *Masson, Paris* 1990.

8. Sugimura T: **Food and cancer**. *Toxicology* 2002, **181-182**:17-21.

9. Ferguson LR, Philpott M: **Nutrition and mutagenesis**. *AnnuRevNutr* 2008, **28**:313-329.

10. Abnet CC: **Carcinogenic food contaminants**. *Cancer Invest* 2007, **25**(3):189-196.

11. Qian GS, Ross RK, Yu MC, Yuan JM, Gao YT, Henderson BE, Wogan GN, Groopman JD: **A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China**. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 1994, **3**(1):3-10.

12. Bulatao-Jayme J, Almero EM, Castro MC, Jardeleza MT, Salamat LA: **A case-control dietary study of primary liver cancer risk from aflatoxin exposure**. *International journal of epidemiology* 1982, **11**(2):112-119.

13. Lunn RM, Zhang YJ, Wang LY, Chen CJ, Lee PH, Lee CS, Tsai WY, Santella RM: **p53 mutations, chronic hepatitis B virus infection, and aflatoxin exposure in hepatocellular carcinoma in Taiwan**. *Cancer research* 1997, **57**(16):3471-3477.

14. Zheng W, Lee SA: **Well-done meat intake, heterocyclic amine exposure, and cancer risk**. *Nutrition and cancer* 2009, **61**(4):437-446.

15. Chen CH, Wang MH, Wang JH, Hung CH, Hu TH, Lee SC, Tung HD, Lee CM, Changchien CS, Chen PF *et al*: **Aflatoxin exposure and hepatitis C virus in advanced liver disease in a hepatitis C virus endemic area in Taiwan**. *The American journal of tropical medicine and hygiene* 2007, **77**(4):747-752.

16. Sun CA, Wu DM, Wang LY, Chen CJ, You SL, Santella RM: **Determinants of formation of aflatoxin-albumin adducts: a seven-township study in Taiwan**. *British journal of cancer* 2002, **87**(9):966-970.

17. Kirk GD, Lesi OA, Mendy M, Szymanska K, Whittle H, Goedert JJ, Hainaut P, Montesano R: **249(ser) TP53 mutation in plasma DNA, hepatitis B viral infection, and risk of hepatocellular carcinoma**. *Oncogene* 2005, **24**(38):5858-5867.

18. Sanyal AJ, Yoon SK, Lencioni R: **The etiology of hepatocellular carcinoma and consequences for treatment**. *The oncologist* 2010, **15 Suppl 4**:14-22.

19. Smela ME, Currier SS, Bailey EA, Essigmann JM: **The chemistry and biology of aflatoxin B(1): from mutational spectrometry to carcinogenesis**. *Carcinogenesis* 2001, **22**(4):535-545.

20. Navarro A, Munoz SE, Lantieri MJ, del Pilar Diaz M, Cristaldo PE, de Fabro SP, Eynard AR: **Meat cooking habits and risk of colorectal cancer in Cordoba, Argentina**. *Nutrition* 2004, **20**(10):873-877.

21. Murtaugh MA, Ma KN, Sweeney C, Caan BJ, Slattery ML: **Meat consumption patterns and preparation, genetic variants of metabolic enzymes, and their association with rectal cancer in men and women**. *The Journal of nutrition* 2004, **134**(4):776-784.

22. Rohrmann S, Hermann S, Linseisen J: **Heterocyclic aromatic amine intake increases colorectal adenoma risk: findings from a prospective European cohort study**. *The American journal of clinical nutrition* 2009, **89**(5):1418-1424.

23. Butler LM, Sinha R, Millikan RC, Martin CF, Newman B, Gammon MD, Ammerman AS, Sandler RS: **Heterocyclic amines, meat intake, and association with colon cancer in a population-based study**. *American journal of epidemiology* 2003, **157**(5):434-445.

24. Nowell S, Coles B, Sinha R, MacLeod S, Luke Ratnasinghe D, Stotts C, Kadlubar FF, Ambrosone CB, Lang NP: **Analysis of total meat intake and exposure to individual heterocyclic amines in a case-control study of colorectal cancer: contribution of metabolic variation to risk**. *Mutation research* 2002, **506-507**:175-185.

25. Steck SE, Gaudet MM, Eng SM, Britton JA, Teitelbaum SL, Neugut AI, Santella RM, Gammon MD: **Cooked meat and risk of breast cancer--lifetime versus recent dietary intake**. *Epidemiology* 2007, **18**(3):373-382.

26. Sinha R: **An epidemiologic approach to studying heterocyclic amines**. *Mutation research* 2002, **506-507**:197-204.

27. Delfino RJ, Sinha R, Smith C, West J, White E, Lin HJ, Liao SY, Gim JS, Ma HL, Butler J *et al*: **Breast cancer, heterocyclic aromatic amines from meat and N-acetyltransferase 2 genotype**. *Carcinogenesis* 2000, **21**(4):607-615.

28. Koutros S, Cross AJ, Sandler DP, Hoppin JA, Ma X, Zheng T, Alavanja MC, Sinha R: **Meat and meat mutagens and risk of prostate cancer in the Agricultural Health Study**. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2008, **17**(1):80-87.

29. Rovito PM, Jr., Morse PD, Spinek K, Newman N, Jones RF, Wang CY, Haas GP: **Heterocyclic amines and genotype of N-acetyltransferases as risk factors for prostate cancer**. *Prostate cancer and prostatic diseases* 2005, **8**(1):69-74.

30. Cross AJ, Peters U, Kirsh VA, Andriole GL, Reding D, Hayes RB, Sinha R: **A prospective study of meat and meat mutagens and prostate cancer risk**. *Cancer research* 2005, **65**(24):11779-11784.

31. Li D, Day RS, Bondy ML, Sinha R, Nguyen NT, Evans DB, Abbruzzese JL, Hassan MM: **Dietary mutagen exposure and risk of pancreatic cancer**. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2007, **16**(4):655-661.

32. Stolzenberg-Solomon RZ, Cross AJ, Silverman DT, Schairer C, Thompson FE, Kipnis V, Subar AF, Hollenbeck A, Schatzkin A, Sinha R: **Meat and meat-mutagen intake and pancreatic cancer risk in the NIH-AARP cohort**. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2007, **16**(12):2664-2675.

33. Anderson KE, Sinha R, Kulldorff M, Gross M, Lang NP, Barber C, Harnack L, DiMagno E, Bliss R, Kadlubar FF: **Meat intake and cooking techniques: associations with pancreatic cancer**. *Mutation research* 2002, **506-507**:225-231.

34. Goldman R, Shields PG: **Food mutagens**. *The Journal of nutrition* 2003, **133 Suppl 3**:965S-973S.

35. Wogan GN, Hecht SS, Felton JS, Conney AH, Loeb LA: **Environmental and chemical carcinogenesis**. *SeminCancer Biol* 2004, **14**(6):473-486.

36. Probst-Hensch NM, Sinha R, Longnecker MP, Witte JS, Ingles SA, Frankl HD, Lee ER, Haile RW: **Meat preparation and colorectal adenomas in a large sigmoidoscopy-based case-control study in California (United States)**. *Cancer causes & control : CCC* 1997, **8**(2):175-183.

37. Giovannucci E, Rimm EB, Stampfer MJ, Colditz GA, Ascherio A, Willett WC: **Intake of fat, meat, and fiber in relation to risk of colon cancer in men**. *Cancer research* 1994, **54**(9):2390-2397.

38. Jagerstad M, Skog K: **Genotoxicity of heat-processed foods**. *Mutation research* 2005, **574**(1-2):156-172.

39. Bartsch H, Montesano R: **Relevance of nitrosamines to human cancer**. *Carcinogenesis* 1984, **5**(11):1381-1393.

40. Mirvish SS: **Role of N-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC**. *Cancer letters* 1995, **93**(1):17-48.

41. Lee SA: **Gene-diet interaction on cancer risk in epidemiological studies**. *JPrevMedPublic Health* 2009, **42**(6):360-370.

42. Muecke R, Schomburg L, Buentzel J, Kisters K, Micke O: **Selenium or no selenium--that is the question in tumor patients: a new controversy**. *IntegrCancer Ther* 2010, **9**(2):136-141.

43. Richie JP, Jr., Muscat JE, Ellison I, Calcagnotto A, Kleinman W, El-Bayoumy K: **Association of selenium status and blood glutathione concentrations in blacks and whites**. *NutrCancer* 2011, **63**(3):367-375.

44. Gil F, Pla A: **Biomarkers as biological indicators of xenobiotic exposure**. *Journal of applied toxicology : JAT* 2001, **21**(4):245-255.

45. Manno M, Viau C, Cocker J, Colosio C, Lowry L, Mutti A, Nordberg M, Wang S: **Biomonitoring for occupational health risk assessment (BOHRA)**. *Toxicology letters* 2010, **192**(1):3-16.

46. Watson WP, Mutti A: **Role of biomarkers in monitoring exposures to chemicals: present position, future prospects**. *Biomarkers : biochemical indicators of exposure, response, and susceptibility to chemicals* 2004, **9**(3):211-242.

47. Silins I, Hogberg J: **Combined toxic exposures and human health: biomarkers of exposure and effect**. *International journal of environmental research and public health* 2011, **8**(3):629-647.

48. Rueff J, Teixeira JP, Santos LS, Gaspar JF: **Genetic effects and biotoxicity monitoring of occupational styrene exposure**. *Clinica chimica acta; international journal of clinical chemistry* 2009, **399**(1-2):8-23.

49. Koivisto P, Peltonen K: **Analytical methods in DNA and protein adduct analysis**. *Analytical and bioanalytical chemistry* 2010, **398**(6):2563-2572.

50. Stone MP, Huang H, Brown KL, Shanmugam G: **Chemistry and structural biology of DNA damage and biological consequences**. *Chemistry & biodiversity* 2011, **8**(9):1571-1615.

51. Maniere I, Godard T, Doerge DR, Churchwell MI, Guffroy M, Laurentie M, Poul JM: **DNA damage and DNA adduct formation in rat tissues following oral administration of acrylamide**. *Mutation research* 2005, **580**(1-2):119-129.

52. Angerer J, Ewers U, Wilhelm M: **Human biomonitoring: state of the art**. *International journal of hygiene and environmental health* 2007, **210**(3-4):201-228.

53. Besaratinia A, Pfeifer GP: **Genotoxicity of acrylamide and glycidamide**. *JNatlCancer Inst* 2004, **96**(13):1023-1029.

54. Carere A: **Genotoxicity and carcinogenicity of acrylamide: a critical review**. *AnnIstSuperSanita* 2006, **42**(2):144-155.

55. Bonassi S, Znaor A, Ceppi M, Lando C, Chang WP, Holland N, Kirsch-Volders M, Zeiger E, Ban S, Barale R *et al*: **An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans**. *Carcinogenesis* 2007, **28**(3):625-631.

56. El-Zein R, Vral A, Etzel CJ: **Cytokinesis-blocked micronucleus assay and cancer risk assessment**. *Mutagenesis* 2011, **26**(1):101-106.

57. Suspiro A, Prista J: **Biomarkers of occupational exposure do anticancer agents: a minireview**. *Toxicology letters* 2011, **207**(1):42-52.

58. Bonassi S, El-Zein R, Bolognesi C, Fenech M: **Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies**. *Mutagenesis* 2011, **26**(1):93-100.

59. Iarmarcovai G, Bonassi S, Botta A, Baan RA, Orsiere T: **Genetic polymorphisms and micronucleus formation: a review of the literature**. *MutatRes* 2008, **658**(3):215-233.

60. Mateuca R, Lombaert N, Aka PV, Decordier I, Kirsch-Volders M: **Chromosomal changes: induction, detection methods and applicability in human biomonitoring**. *Biochimie* 2006, **88**(11):1515-1531.

61. Pavanello S, Clonfero E: **Biological indicators of genotoxic risk and metabolic polymorphisms**. *Mutation research* 2000, **463**(3):285-308.

62. Hagmar L, Brogger A, Hansteen IL, Heim S, Hogstedt B, Knudsen L, Lambert B, Linnainmaa K, Mitelman F, Nordenson I *et al*: **Cancer risk in humans predicted by increased levels of chromosomal aberrations in lymphocytes: Nordic study group on the health risk of chromosome damage**. *Cancer research* 1994, **54**(11):2919-2922.

63. Bonassi S, Abbondandolo A, Camurri L, Dal Pra L, De Ferrari M, Degrassi F, Forni A, Lamberti L, Lando C, Padovani P *et al*: **Are chromosome aberrations in circulating lymphocytes predictive of future cancer onset in humans? Preliminary results of an Italian cohort study**. *Cancer genetics and cytogenetics* 1995, **79**(2):133-135.

64. Garcia-Sagredo JM: **Fifty years of cytogenetics: a parallel view of the evolution of cytogenetics and genotoxicology**. *Biochimica et biophysica acta* 2008, **1779**(6-7):363-375.

65. Wilson DM, 3rd, Thompson LH: **Molecular mechanisms of sister-chromatid exchange**. *Mutation research* 2007, **616**(1-2):11-23.

66. Norppa H, Bonassi S, Hansteen IL, Hagmar L, Stromberg U, Rossner P, Boffetta P, Lindholm C, Gundy S, Lazutka J *et al*: **Chromosomal aberrations and SCEs as biomarkers of cancer risk**. *Mutation research* 2006, **600**(1-2):37-45.

67. Collins AR: **The comet assay for DNA damage and repair: principles, applications, and limitations**. *Molecular biotechnology* 2004, **26**(3):249-261.

68. Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR, Escobar P, Honma M, Kumaravel TS, Nakajima M *et al*: **Fourth International Workgroup on Genotoxicity testing: results of the in vivo Comet assay workgroup**. *Mutation research* 2007, **627**(1):31-35.

69. Collins AR, Oscoz AA, Brunborg G, Gaivao I, Giovannelli L, Kruszewski M, Smith CC, Stetina R: **The comet assay: topical issues**. *Mutagenesis* 2008, **23**(3):143-151.

70. Faust F, Kassie F, Knasmuller S, Boedecker RH, Mann M, Mersch-Sundermann V: **The use of the alkaline comet assay with lymphocytes in human biomonitoring studies**. *Mutation research* 2004, **566**(3):209-229.

71. Moller P, Knudsen LE, Loft S, Wallin H: **The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors**. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2000, **9**(10):1005-1015.

72. Jancova P, Anzenbacher P, Anzenbacherova E: **Phase II drug metabolizing enzymes**. *Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia* 2010, **154**(2):103-116.

73. Duale N, Bjellaas T, Alexander J, Becher G, Haugen M, Paulsen JE, Frandsen H, Olesen PT, Brunborg G: **Biomarkers of human exposure to acrylamide and relation to polymorphisms in metabolizing genes**. *ToxicolSci* 2009, **108**(1):90-99.

74. Paulsson B, Rannug A, Henderson AP, Golding BT, Tornqvist M, Warholm M: **In vitro studies of the influence of glutathione transferases and epoxide hydrolase on the detoxification of acrylamide and glycidamide in blood**. *MutatRes* 2005, **580**(1-2):53-59.

75. Chefson A, Auclair K: **Progress towards the easier use of P450 enzymes**. *Molecular bioSystems* 2006, **2**(10):462-469.

76. Autrup H: **Genetic polymorphisms in human xenobiotica metabolizing enzymes as susceptibility factors in toxic response**. *Mutation research* 2000, **464**(1):65-76.

77. Teixeira JP, Gaspar J, Martinho G, Silva S, Rodrigues S, Mayan O, Martin E, Farmer PB, Rueff J: **Aromatic DNA adduct levels in coke oven workers: correlation with polymorphisms in genes GSTP1, GSTM1, GSTT1 and CYP1A1**. *MutatRes* 2002, **517**(1-2):147-155.

78. Casson AG, Zheng Z, Chiasson D, MacDonald K, Riddell DC, Guernsey JR, Guernsey DL, McLaughlin J: **Associations between genetic polymorphisms of Phase I and II metabolizing enzymes, p53 and susceptibility to esophageal adenocarcinoma**. *Cancer detection and prevention* 2003, **27**(2):139-146.

79. Smits KM, Schouten LJ, van Dijk BA, van Houwelingen K, Hulsbergen-van de Kaa CA, Kiemeney LA, Goldbohm RA, Oosterwijk E, van den Brandt PA: **Polymorphisms in genes related to activation or detoxification of carcinogens might interact with smoking to increase renal cancer risk: results from The Netherlands Cohort Study on diet and cancer**. *World journal of urology* 2008, **26**(1):103-110.

80. Bozina N, Bradamante V, Lovric M: **Genetic polymorphism of metabolic enzymes P450 (CYP) as a susceptibility factor for drug response, toxicity, and cancer risk**. *Arhiv za higijenu rada i toksikologiju* 2009, **60**(2):217-242.

81. Zhan P, Wang Q, Qian Q, Wei SZ, Yu LK: **CYP1A1 MspI and exon7 gene polymorphisms and lung cancer risk: an updated meta-analysis and review**. *Journal of experimental & clinical cancer research : CR* 2011, **30**:99.

82. Vijayakrishnan J, Houlston RS: **Candidate gene association studies and risk of childhood acute lymphoblastic leukemia: a systematic review and meta-analysis**. *Haematologica* 2010, **95**(8):1405-1414.

83. Landi S, Gemignani F, Moreno V, Gioia-Patricola L, Chabrier A, Guino E, Navarro M, de Oca J, Capella G, Canzian F: **A comprehensive analysis of phase I and phase II metabolism gene polymorphisms and risk of colorectal cancer**. *Pharmacogenetics and genomics* 2005, **15**(8):535-546.

84. Chen J, Cheng M, Yi L, Jiang CB: **Relationship Between CYP1A1 Genetic Polymorphisms and Renal Cancer in China**. *Asian Pacific journal of cancer prevention : APJCP* 2011, **12**(9):2163-2166.

85. Hiyama T, Yoshihara M, Tanaka S, Chayama K: **Genetic polymorphisms and esophageal cancer risk**. *International journal of cancer Journal international du cancer* 2007, **121**(8):1643-1658.

86. Settels E, Bernauer U, Palavinskas R, Klaffke HS, Gundert-Remy U, Appel KE: **Human CYP2E1 mediates the formation of glycidamide from acrylamide**. *Archives of toxicology* 2008, **82**(10):717-727.

87. Wang Y, Yang H, Li L, Wang H, Zhang C, Yin G, Zhu B: **Association between CYP2E1 genetic polymorphisms and lung cancer risk: a meta-analysis**. *Eur J Cancer* 2010, **46**(4):758-764.

88. Paulsson B, Warholm M, Rannug A, Tornqvist M: **In vitro studies of the influence of certain enzymes on the detoxification of acrylamide and glycidamide in blood**. *AdvExpMedBiol* 2005, **561**:127-133.

89. Wu MS, Chen CJ, Lin MT, Wang HP, Shun CT, Sheu JC, Lin JT: **Genetic polymorphisms of cytochrome p450 2E1, glutathione S-transferase M1 and T1, and susceptibility to gastric carcinoma in Taiwan**. *International journal of colorectal disease* 2002, **17**(5):338-343.

90. Kiyohara C, Yoshimasu K, Takayama K, Nakanishi Y: **EPHX1 polymorphisms and the risk of lung cancer: a HuGE review**. *Epidemiology* 2006, **17**(1):89-99.

91. Silva MC, Gaspar J, Duarte SI, Faber A, Rueff J: **GSTM1, GSTT1, and GSTP1 genotypes and the genotoxicity of hydroquinone in human lymphocytes**. *EnvironMolMutagen* 2004, **43**(4):258-264.

92. Zhang ZJ, Hao K, Shi R, Zhao G, Jiang GX, Song Y, Xu X, Ma J: **Glutathione S-transferase M1 (GSTM1) and glutathione S-transferase T1 (GSTT1) null polymorphisms, smoking, and their interaction in oral cancer: a HuGE review and meta-analysis**. *American journal of epidemiology* 2011, **173**(8):847-857.

93. Carlsten C, Sagoo GS, Frodsham AJ, Burke W, Higgins JP: **Glutathione S-transferase M1 (GSTM1) polymorphisms and lung cancer: a literature-based systematic HuGE review and meta-analysis**. *American journal of epidemiology* 2008, **167**(7):759-774.

94. Gao Y, Cao Y, Tan A, Liao C, Mo Z, Gao F: **Glutathione S-transferase M1 polymorphism and sporadic colorectal cancer risk: An updating meta-analysis and HuGE review of 36 case-control studies**. *Annals of epidemiology* 2010, **20**(2):108-121.

95. Cote ML, Chen W, Smith DW, Benhamou S, Bouchardy C, Butkiewicz D, Fong KM, Gene M, Hirvonen A, Kiyohara C *et al*: **Meta- and pooled analysis of GSTP1 polymorphism and lung cancer: a HuGE-GSEC review**. *American journal of epidemiology* 2009, **169**(7):802-814.

96. Kellen E, Hemelt M, Broberg K, Golka K, Kristensen VN, Hung RJ, Matullo G, Mittal RD, Porru S, Povey A *et al*: **Pooled analysis and meta-analysis of the glutathione S-transferase P1 Ile 105Val polymorphism and bladder cancer: a HuGE-GSEC review**. *American journal of epidemiology* 2007, **165**(11):1221-1230.

97. Ramos DL, Gaspar JF, Pingarilho M, Gil OM, Fernandes AS, Rueff J, Oliveira NG: **Genotoxic effects of doxorubicin in cultured human lymphocytes with different glutathione S-transferase genotypes**. *MutatRes* 2011, **724**(1-2):28-34.

98. Silva SN, Azevedo AP, Teixeira V, Pina JE, Rueff J, Gaspar JF: **The role of GSTA2 polymorphisms and haplotypes in breast cancer susceptibility: a case-control study in the Portuguese population**. *OncolRep* 2009, **22**(3):593-598.

99. Tetlow N, Board PG: **Functional polymorphism of human glutathione transferase A2**. *Pharmacogenetics* 2004, **14**(2):111-116.

100. Coles BF, Kadlubar FF: **Human alpha class glutathione S-transferases: genetic polymorphism, expression, and susceptibility to disease**. *Methods in enzymology* 2005, **401**:9-42.

101. Andonova IE, Justenhoven C, Winter S, Hamann U, Baisch C, Rabstein S, Spickenheuer A, Harth V, Pesch B, Bruning T *et al*: **No evidence for glutathione S-transferases GSTA2, GSTM2, GSTO1, GSTO2, and GSTZ1 in breast cancer risk**. *Breast cancer research and treatment* 2010, **121**(2):497-502.

102. Ning B, Wang C, Morel F, Nowell S, Ratnasinghe DL, Carter W, Kadlubar FF, Coles B: **Human glutathione S-transferase A2 polymorphisms: variant expression, distribution in prostate cancer cases/controls and a novel form**. *Pharmacogenetics* 2004, **14**(1):35-44.

103. Gemignani F, Landi S, Szeszenia-Dabrowska N, Zaridze D, Lissowska J, Rudnai P, Fabianova E, Mates D, Foretova L, Janout V *et al*: **Development of lung cancer before the age of 50: the role of xenobiotic metabolizing genes**. *Carcinogenesis* 2007, **28**(6):1287-1293.

104. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S: **Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints**. *AnnuRevBiochem* 2004, **73**:39-85.

105. Hegde ML, Hazra TK, Mitra S: **Early steps in the DNA base excision/single-strand interruption repair pathway in mammalian cells**. *Cell Res* 2008, **18**(1):27-47.

106. Hoeijmakers JH: **Genome maintenance mechanisms for preventing cancer**. *Nature* 2001, **411**(6835):366-374.

107. Madhusudan S, Middleton MR: **The emerging role of DNA repair proteins as predictive, prognostic and therapeutic targets in cancer**. *Cancer TreatRev* 2005, **31**(8):603-617.

108. Mohrenweiser HW, Wilson DM, III, Jones IM: **Challenges and complexities in estimating both the functional impact and the disease risk associated with the extensive genetic variation in human DNA repair genes**. *MutatRes* 2003, **526**(1-2):93-125.

109. Christmann M, Tomicic MT, Roos WP, Kaina B: **Mechanisms of human DNA repair: an update**. *Toxicology* 2003, **193**(1-2):3-34.

110. Braithwaite E, Wu X, Wang Z: **Repair of DNA lesions: mechanisms and relative repair efficiencies**. *MutatRes* 1999, **424**(1-2):207-219.

111. Houtgraaf JH, Versmissen J, van der Giessen WJ: **A concise review of DNA damage checkpoints and repair in mammalian cells**. *CardiovascRevascMed* 2006, **7**(3):165-172.

112. Al-Tassan N, Chmiel NH, Maynard J, Fleming N, Livingston AL, Williams GT, Hodges AK, Davies DR, David SS, Sampson JR *et al*: **Inherited variants of MYH associated with somatic G:C-->T:A mutations in colorectal tumors**. *Nature genetics* 2002, **30**(2):227-232.

113. Isidro G, Laranjeira F, Pires A, Leite J, Regateiro F, Castro e Sousa F, Soares J, Castro C, Giria J, Brito MJ *et al*: **Germline MUTYH (MYH) mutations in Portuguese individuals with multiple colorectal adenomas**. *Human mutation* 2004, **24**(4):353-354.

114. Wilson DM, 3rd, Bohr VA: **The mechanics of base excision repair, and its relationship to aging and disease**. *DNA repair* 2007, **6**(4):544-559.

115. Jascur T, Boland CR: **Structure and function of the components of the human DNA mismatch repair system**. *IntJCancer* 2006, **119**(9):2030-2035.

116. Sies H: **Oxidative stress: oxidants and antioxidants**. *Experimental physiology* 1997, **82**(2):291-295.

117. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J: **Free radicals and antioxidants in normal physiological functions and human disease**. *The international journal of biochemistry & cell biology* 2007, **39**(1):44-84.

118. Halliwell B: **Biochemistry of oxidative stress**. *Biochemical Society transactions* 2007, **35**(Pt 5):1147-1150.

119. Mates JM: **Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology**. *Toxicology* 2000, **153**(1-3):83-104.

120. Mates JM, Perez-Gomez C, Nunez de Castro I: **Antioxidant enzymes and human diseases**. *Clinical biochemistry* 1999, **32**(8):595-603.

121. Day BJ: **Catalytic antioxidants: a radical approach to new therapeutics**. *Drug discovery today* 2004, **9**(13):557-566.

122. Ma Q: **Transcriptional responses to oxidative stress: pathological and toxicological implications**. *Pharmacology & therapeutics* 2010, **125**(3):376-393.

123. Weinberg F, Chandel NS: **Reactive oxygen species-dependent signaling regulates cancer**. *Cellular and molecular life sciences : CMLS* 2009, **66**(23):3663-3673.

124. Mena S, Ortega A, Estrela JM: **Oxidative stress in environmental-induced carcinogenesis**. *Mutation research* 2009, **674**(1-2):36-44.

125. Finkel T, Holbrook NJ: **Oxidants, oxidative stress and the biology of ageing**. *Nature* 2000, **408**(6809):239-247.

126. Goetz ME, Luch A: **Reactive species: a cell damaging rout assisting to chemical carcinogens**. *Cancer letters* 2008, **266**(1):73-83.

127. Droge W: **Free radicals in the physiological control of cell function**. *Physiological reviews* 2002, **82**(1):47-95.

128. Friedman M: **Chemistry, biochemistry, and safety of acrylamide. A review**. *JAgricFood Chem* 2003, **51**(16):4504-4526.

129. Parzefall W: **Minireview on the toxicity of dietary acrylamide**. *Food ChemToxicol* 2008, **46**(4):1360-1364.

130. Ruden C: **Acrylamide and cancer risk--expert risk assessments and the public debate**. *Food ChemToxicol* 2004, **42**(3):335-349.

131. Rice JM: **The carcinogenicity of acrylamide**. *MutatRes* 2005, **580**(1-2):3-20.

132. Mucci LA, Wilson KM: **Acrylamide intake through diet and human cancer risk**. *JAgricFood Chem* 2008, **56**(15):6013-6019.

133. IARC: **Acrylamide**. In: *Acrylamide In IARC monographs on the evaluation of carcinogenic risk to humans Volume 60*, edn.: International Agency for Research on Cancer, Lyon; 1994: 389-433.

134. Mottram DS, Wedzicha BL, Dodson AT: **Acrylamide is formed in the Maillard reaction**. *Nature* 2002, **419**(6906):448-449.

135. Stadler RH, Blank I, Varga N, Robert F, Hau J, Guy PA, Robert MC, Riediker S: **Acrylamide from Maillard reaction products**. *Nature* 2002, **419**(6906):449-450.

136. Heatox: **Heat-generated food toxicants: identification, characterisation and risk minimization. Project no. 506820 - Final Report**. *Food Quality and Safety, Lund* 2007: 1-77.

137. Keramat J, LeBail A, Prost C, Soltanizadeh N: **Acrylamide in Foods: Chemistry and Analysis. A review article.** *Food Bioprocess Technol* 2011, **4**:340-363.

138. Klaunig JE: **Acrylamide carcinogenicity**. *JAgricFood Chem* 2008, **56**(15):5984-5988.

139. FAO/WHO: **Joint FAO/WHO Expert Commitee on Food Additives (JECFA) - Summary and conclusions of the sixty-fourth meeting**. *Food and Drug Administration/World Health Organization, Rome* 2005: 1-47.

140. Hartmann EC, Boettcher MI, Schettgen T, Fromme H, Drexler H, Angerer J: **Hemoglobin adducts and mercapturic acid excretion of acrylamide and glycidamide in one study population**. *Journal of agricultural and food chemistry* 2008, **56**(15):6061-6068.

141. Dybing E, Sanner T: **Risk assessment of acrylamide in foods**. *ToxicolSci* 2003, **75**(1):7-15.

142. Petersen BJ, Tran N: **Exposure to acrylamide: placing exposure in context**. *Advances in experimental medicine and biology* 2005, **561**:63-76.

143. Friedman M, Levin CE: **Review of methods for the reduction of dietary content and toxicity of acrylamide**. *Journal of agricultural and food chemistry* 2008, **56**(15):6113-6140.

144. EFSA: **Scientific report of European Food Safety Authority (EFSA) - Results on acrylamide levels in food from monitoring year 2008**. *EFSA Journal* 2010(8):1-31.

145. Tareke E, Rydberg P, Karlsson P, Eriksson S, Tornqvist M: **Analysis of acrylamide, a carcinogen formed in heated foodstuffs**. *Journal of agricultural and food chemistry* 2002, **50**(17):4998-5006.

146. Besaratinia A, Pfeifer GP: **DNA adduction and mutagenic properties of acrylamide**. *Mutation research* 2005, **580**(1-2):31-40.

147. Bergmark E, Calleman CJ, He F, Costa LG: **Determination of hemoglobin adducts in humans occupationally exposed to acrylamide**. *Toxicology and applied pharmacology* 1993, **120**(1):45-54.

148. Fuhr U, Boettcher MI, Kinzig-Schippers M, Weyer A, Jetter A, Lazar A, Taubert D, Tomalik-Scharte D, Pournara P, Jakob V *et al*: **Toxicokinetics of acrylamide in humans after ingestion of a defined dose in a test meal to improve risk assessment for acrylamide carcinogenicity**. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2006, **15**(2):266-271.

149. Fennell TR, Sumner SC, Snyder RW, Burgess J, Friedman MA: **Kinetics of elimination of urinary metabolites of acrylamide in humans**. *Toxicological sciences : an official journal of the Society of Toxicology* 2006, **93**(2):256-267.

150. Doerge DR, Young JF, McDaniel LP, Twaddle NC, Churchwell MI: **Toxicokinetics of acrylamide and glycidamide in B6C3F1 mice**. *Toxicology and applied pharmacology* 2005, **202**(3):258-267.

151. Doerge DR, Young JF, McDaniel LP, Twaddle NC, Churchwell MI: **Toxicokinetics of acrylamide and glycidamide in Fischer 344 rats**. *Toxicology and applied pharmacology* 2005, **208**(3):199-209.

152. Sorgel F, Weissenbacher R, Kinzig-Schippers M, Hofmann A, Illauer M, Skott A, Landersdorfer C: **Acrylamide: increased concentrations in homemade food and first evidence of its variable absorption from food, variable metabolism and placental and breast milk transfer in humans**. *Chemotherapy* 2002, **48**(6):267-274.

153. Gargas ML, Kirman CR, Sweeney LM, Tardiff RG: **Acrylamide: Consideration of species differences and nonlinear processes in estimating risk and safety for human ingestion**. *Food ChemToxicol* 2009, **47**(4):760-768.

154. Doroshyenko O, Fuhr U, Kunz D, Frank D, Kinzig M, Jetter A, Reith Y, Lazar A, Taubert D, Kirchheiner J *et al*: **In vivo role of cytochrome P450 2E1 and glutathione-S-transferase activity for acrylamide toxicokinetics in humans**. *Cancer EpidemiolBiomarkers Prev* 2009, **18**(2):433-443.

155. Kopp EK, Dekant W: **Toxicokinetics of acrylamide in rats and humans following single oral administration of low doses**. *Toxicology and applied pharmacology* 2009, **235**(2):135-142.

156. Boettcher MI, Bolt HM, Drexler H, Angerer J: **Excretion of mercapturic acids of acrylamide and glycidamide in human urine after single oral administration of deuterium-labelled acrylamide**. *Archives of toxicology* 2006, **80**(2):55-61.

157. Vesper HW, Slimani N, Hallmans G, Tjonneland A, Agudo A, Benetou V, Bingham S, Boeing H, Boutron-Ruault MC, Bueno-de-Mesquita HB *et al*: **Cross-sectional study on acrylamide hemoglobin adducts in subpopulations from the European Prospective Investigation into Cancer and Nutrition (EPIC) Study**. *Journal of agricultural and food chemistry* 2008, **56**(15):6046-6053.

158. Vikstrom AC, Wilson KM, Paulsson B, Athanassiadis I, Gronberg H, Adami HO, Adolfsson J, Mucci LA, Balter K, Tornqvist M: **Alcohol influence on acrylamide to glycidamide metabolism assessed with hemoglobin-adducts and questionnaire data**. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 2010, **48**(3):820-824.

159. Bergmark E: **Hemoglobin adducts of acrylamide and acrylonitrile in laboratory workers, smokers and nonsmokers**. *Chemical research in toxicology* 1997, **10**(1):78-84.

160. Hagmar L, Wirfalt E, Paulsson B, Tornqvist M: **Differences in hemoglobin adduct levels of acrylamide in the general population with respect to dietary intake, smoking habits and gender**. *Mutation research* 2005, **580**(1-2):157-165.

161. Schettgen T, Weiss T, Drexler H, Angerer J: **A first approach to estimate the internal exposure to acrylamide in smoking and non-smoking adults from Germany**. *International journal of hygiene and environmental health* 2003, **206**(1):9-14.

162. Fennell TR, Snyder RW, Krol WL, Sumner SC: **Comparison of the hemoglobin adducts formed by administration of N-methylolacrylamide and acrylamide to rats**. *Toxicological sciences : an official journal of the Society of Toxicology* 2003, **71**(2):164-175.

163. Fennell TR, Friedman MA: **Comparison of acrylamide metabolism in humans and rodents**. *Advances in experimental medicine and biology* 2005, **561**:109-116.

164. Gamboa da Costa G, Churchwell MI, Hamilton LP, Von Tungeln LS, Beland FA, Marques MM, Doerge DR: **DNA adduct formation from acrylamide via conversion to glycidamide in adult and neonatal mice**. *Chemical research in toxicology* 2003, **16**(10):1328-1337.

165. Solomon JJ, Fedyk J, Mukai F, Segal A: **Direct alkylation of 2'-deoxynucleosides and DNA following in vitro reaction with acrylamide**. *Cancer research* 1985, **45**(8):3465-3470.

166. Segerback D, Calleman CJ, Schroeder JL, Costa LG, Faustman EM: **Formation of N-7-(2-carbamoyl-2-hydroxyethyl)guanine in DNA of the mouse and the rat following intraperitoneal administration of [14C]acrylamide**. *Carcinogenesis* 1995, **16**(5):1161-1165.

167. Atay NZ, Çalgan D, Özakat E, Varnali T: **Acrylamide and glycidamide adducts of Guanine**. *Theochem* 2005, **728**:29-251.

168. Ghanayem BI, Witt KL, Kissling GE, Tice RR, Recio L: **Absence of acrylamide-induced genotoxicity in CYP2E1-null mice: evidence consistent with a glycidamide-mediated effect**. *Mutation research* 2005, **578**(1-2):284-297.

169. Blasiak J, Gloc E, Wozniak K, Czechowska A: **Genotoxicity of acrylamide in human lymphocytes**. *Chemico-biological interactions* 2004, **149**(2-3):137-149.

170. Koyama N, Sakamoto H, Sakuraba M, Koizumi T, Takashima Y, Hayashi M, Matsufuji H, Yamagata K, Masuda S, Kinae N *et al*: **Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells**. *Mutation research* 2006, **603**(2):151-158.

171. Mei N, Hu J, Churchwell MI, Guo L, Moore MM, Doerge DR, Chen T: **Genotoxic effects of acrylamide and glycidamide in mouse lymphoma cells**. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 2008, **46**(2):628-636.

172. Baum M, Fauth E, Fritzen S, Herrmann A, Mertes P, Merz K, Rudolphi M, Zankl H, Eisenbrand G: **Acrylamide and glycidamide: genotoxic effects in V79-cells and human blood**. *Mutation research* 2005, **580**(1-2):61-69.

173. Dearfield KL, Douglas GR, Ehling UH, Moore MM, Sega GA, Brusick DJ: **Acrylamide: a review of its genotoxicity and an assessment of heritable genetic risk**. *MutatRes* 1995, **330**(1-2):71-99.

174. Mei N, McDaniel LP, Dobrovolsky VN, Guo X, Shaddock JG, Mittelstaedt RA, Azuma M, Shelton SD, McGarrity LJ, Doerge DR *et al*: **The genotoxicity of acrylamide and glycidamide in big blue rats**. *Toxicological sciences : an official journal of the Society of Toxicology* 2010, **115**(2):412-421.

175. Manjanatha MG, Aidoo A, Shelton SD, Bishop ME, McDaniel LP, Lyn-Cook LE, Doerge DR: **Genotoxicity of acrylamide and its metabolite glycidamide administered in drinking water to male and female Big Blue mice**. *Environmental and molecular mutagenesis* 2006, **47**(1):6-17.

176. Martins C, Oliveira NG, Pingarilho M, Gamboa da CG, Martins V, Marques MM, Beland FA, Churchwell MI, Doerge DR, Rueff J *et al*: **Cytogenetic damage induced by acrylamide and glycidamide in mammalian cells: correlation with specific glycidamide-DNA adducts**. *ToxicolSci* 2007, **95**(2):383-390.

177. Oliveira NG, Pingarilho M, Martins C, Fernandes AS, Vaz S, Martins V, Rueff J, Gaspar JF: **Cytotoxicity and chromosomal aberrations induced by acrylamide in V79 cells: role of glutathione modulators**. *Mutation research* 2009, **676**(1-2):87-92.

178. Tsuda H, Shimizu CS, Taketomi MK, Hasegawa MM, Hamada A, Kawata KM, Inui N: **Acrylamide; induction of DNA damage, chromosomal aberrations and cell transformation without gene mutations**. *Mutagenesis* 1993, **8**(1):23-29.

179. Adler ID, Ingwersen I, Kliesch U, el Tarras A: **Clastogenic effects of acrylamide in mouse bone marrow cells**. *Mutation research* 1988, **206**(3):379-385.

180. Jiang L, Cao J, An Y, Geng C, Qu S, Zhong L: **Genotoxicity of acrylamide in human hepatoma G2 (HepG2) cells**. *Toxicology in vitro : an international journal published in association with BIBRA* 2007, **21**(8):1486-1492.

181. Puppel N, Tjaden Z, Fueller F, Marko D: **DNA strand breaking capacity of acrylamide and glycidamide in mammalian cells**. *Mutation research* 2005, **580**(1-2):71-80.

182. Hansen SH, Olsen AK, Soderlund EJ, Brunborg G: **In vitro investigations of glycidamide-induced DNA lesions in mouse male germ cells and in mouse and human lymphocytes**. *Mutation research* 2010, **696**(1):55-61.

183. Cao J, Liu Y, Jia L, Jiang LP, Geng CY, Yao XF, Kong Y, Jiang BN, Zhong LF: **Curcumin attenuates acrylamide-induced cytotoxicity and genotoxicity in HepG2 cells by ROS scavenging**. *Journal of agricultural and food chemistry* 2008, **56**(24):12059-12063.

184. Paulsson B, Grawe J, Tornqvist M: **Hemoglobin adducts and micronucleus frequencies in mouse and rat after acrylamide or N-methylolacrylamide treatment**. *Mutation research* 2002, **516**(1-2):101-111.

185. Paulsson B, Kotova N, Grawe J, Henderson A, Granath F, Golding B, Tornqvist M: **Induction of micronuclei in mouse and rat by glycidamide, genotoxic metabolite of acrylamide**. *Mutation research* 2003, **535**(1):15-24.

186. Sumner SC, Selvaraj L, Nauhaus SK, Fennell TR: **Urinary metabolites from F344 rats and B6C3F1 mice coadministered acrylamide and acrylonitrile for 1 or 5 days**. *Chemical research in toxicology* 1997, **10**(10):1152-1160.

187. Ghanayem BI, Witt KL, El-Hadri L, Hoffler U, Kissling GE, Shelby MD, Bishop JB: **Comparison of germ cell mutagenicity in male CYP2E1-null and wild-type mice treated with acrylamide: evidence supporting a glycidamide-mediated effect**. *Biology of reproduction* 2005, **72**(1):157-163.

188. Pingarilho M ON, Martins C, Fernandes AS, Lima JP, Rueff J, Gaspar JF: **Genetic polymorphisms in detoxification and DNA repair genes and *in vitro* susceptibility to glycidamide-induced DNA damage**. *Journal of Toxicology and Environmental Health A* 2012(acepted).

189. Johnson KA, Gorzinski SJ, Bodner KM, Campbell RA, Wolf CH, Friedman MA, Mast RW: **Chronic toxicity and oncogenicity study on acrylamide incorporated in the drinking water of Fischer 344 rats**. *Toxicology and applied pharmacology* 1986, **85**(2):154-168.

190. Friedman MA, Dulak LH, Stedham MA: **A lifetime oncogenicity study in rats with acrylamide**. *Fundamental and applied toxicology : official journal of the Society of Toxicology* 1995, **27**(1):95-105.

191. Sobel W, Bond GG, Parsons TW, Brenner FE: **Acrylamide cohort mortality study**. *BrJIndMed* 1986, **43**(11):785-788.

192. Collins JJ, Swaen GM, Marsh GM, Utidjian HM, Caporossi JC, Lucas LJ: **Mortality patterns among workers exposed to acrylamide**. *JOccupMed* 1989, **31**(7):614-617.

193. Marsh GM, Lucas LJ, Youk AO, Schall LC: **Mortality patterns among workers exposed to acrylamide: 1994 follow up**. *Occupational and environmental medicine* 1999, **56**(3):181-190.

194. Marsh GM, Youk AO, Buchanich JM, Kant IJ, Swaen G: **Mortality patterns among workers exposed to acrylamide: updated follow up**. *Journal of occupational and environmental medicine / American College of Occupational and Environmental Medicine* 2007, **49**(1):82-95.

195. Wilson KM, Balter K, Adami HO, Gronberg H, Vikstrom AC, Paulsson B, Tornqvist M, Mucci LA: **Acrylamide exposure measured by food frequency questionnaire and hemoglobin adduct levels and prostate cancer risk in the Cancer of the Prostate in Sweden Study**. *IntJCancer* 2009, **124**(10):2384-2390.

196. Olesen PT, Olsen A, Frandsen H, Frederiksen K, Overvad K, Tjonneland A: **Acrylamide exposure and incidence of breast cancer among postmenopausal women in the Danish Diet, Cancer and Health Study**. *IntJCancer* 2008, **122**(9):2094-2100.

197. Bandarra S: **Avaliação da citotoxicidade e genotoxicidade da glicidamida em células mamárias humanas**. *Mestrado em Controlo de Qualidade e Toxicologia dos Alimentos* 2010.

198. Mucci LA, Dickman PW, Steineck G, Adami HO, Augustsson K: **Dietary acrylamide and cancer of the large bowel, kidney, and bladder: absence of an association in a population-based study in Sweden**. *British journal of cancer* 2003, **88**(1):84-89.

199. Mucci LA, Lindblad P, Steineck G, Adami HO: **Dietary acrylamide and risk of renal cell cancer**. *International journal of cancer Journal international du cancer* 2004, **109**(5):774-776.

200. Mucci LA, Sandin S, Balter K, Adami HO, Magnusson C, Weiderpass E: **Acrylamide intake and breast cancer risk in Swedish women**. *JAMA : the journal of the American Medical Association* 2005, **293**(11):1326-1327.

201. Mucci LA, Adami HO, Wolk A: **Prospective study of dietary acrylamide and risk of colorectal cancer among women**. *International journal of cancer Journal international du cancer* 2006, **118**(1):169-173.

202. Michels KB, Rosner BA, Chumlea WC, Colditz GA, Willett WC: **Preschool diet and adult risk of breast cancer**. *International journal of cancer Journal international du cancer* 2006, **118**(3):749-754.

203. Pelucchi C, Galeone C, Levi F, Negri E, Franceschi S, Talamini R, Bosetti C, Giacosa A, La Vecchia C: **Dietary acrylamide and human cancer**. *International journal of cancer Journal international du cancer* 2006, **118**(2):467-471.

204. Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA: **A prospective study of dietary acrylamide intake and the risk of endometrial, ovarian, and breast cancer**. *Cancer EpidemiolBiomarkers Prev* 2007, **16**(11):2304-2313.

205. Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA: **Dietary acrylamide intake and the risk of renal cell, bladder, and prostate cancer**. *AmJClinNutr* 2008, **87**(5):1428-1438.

206. Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA: **Lung cancer risk in relation to dietary acrylamide intake**. *Journal of the National Cancer Institute* 2009, **101**(9):651-662.

207. Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA: **Dietary acrylamide intake and brain cancer risk**. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2009, **18**(5):1663-1666.

208. Larsson SC, Akesson A, Wolk A: **Long-term dietary acrylamide intake and breast cancer risk in a prospective cohort of Swedish women**. *American journal of epidemiology* 2009, **169**(3):376-381.

209. Larsson SC, Akesson A, Wolk A: **Long-term dietary acrylamide intake and risk of epithelial ovarian cancer in a prospective cohort of Swedish women**. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2009, **18**(3):994-997.

210. Larsson SC, Hakansson N, Akesson A, Wolk A: **Long-term dietary acrylamide intake and risk of endometrial cancer in a prospective cohort of Swedish women**. *International journal of cancer Journal international du cancer* 2009, **124**(5):1196-1199.

211. Larsson SC, Akesson A, Bergkvist L, Wolk A: **Dietary acrylamide intake and risk of colorectal cancer in a prospective cohort of men**. *Eur J Cancer* 2009, **45**(4):513-516.

212. Larsson SC, Akesson A, Wolk A: **Dietary acrylamide intake and prostate cancer risk in a prospective cohort of Swedish men**. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2009, **18**(6):1939-1941.

213. Schouten LJ, Hogervorst JG, Konings EJ, Goldbohm RA, van den Brandt PA: **Dietary acrylamide intake and the risk of head-neck and thyroid cancers: results from the Netherlands Cohort Study**. *American journal of epidemiology* 2009, **170**(7):873-884.

214. Wilson KM, Mucci LA, Cho E, Hunter DJ, Chen WY, Willett WC: **Dietary acrylamide intake and risk of premenopausal breast cancer**. *AmJEpidemiol* 2009, **169**(8):954-961.

215. Burley VJ, Greenwood DC, Hepworth SJ, Fraser LK, de Kok TM, van Breda SG, Kyrtopoulos SA, Botsivali M, Kleinjans J, McKinney PA *et al*: **Dietary acrylamide intake and risk of breast cancer in the UK women's cohort**. *BrJCancer* 2010, **103**(11):1749-1754.

216. Pedersen GS, Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA: **Dietary acrylamide intake and estrogen and progesterone receptor-defined postmenopausal breast cancer risk**. *Breast Cancer ResTreat* 2010, **122**(1):199-210.

217. Wilson KM, Mucci LA, Rosner BA, Willett WC: **A prospective study on dietary acrylamide intake and the risk for breast, endometrial, and ovarian cancers**. *Cancer EpidemiolBiomarkers Prev* 2010, **19**(10):2503-2515.

218. Hirvonen T, Kontto J, Jestoi M, Valsta L, Peltonen K, Pietinen P, Virtanen SM, Sinkko H, Kronberg-Kippila C, Albanes D *et al*: **Dietary acrylamide intake and the risk of cancer among Finnish male smokers**. *Cancer causes & control : CCC* 2010, **21**(12):2223-2229.

219. Lin Y, Lagergren J, Lu Y: **Dietary acrylamide intake and risk of esophageal cancer in a population-based case-control study in Sweden**. *International journal of cancer Journal international du cancer* 2011, **128**(3):676-681.

220. Wilson KM, Giovannucci E, Stampfer MJ, Mucci LA: **Dietary acrylamide and risk of prostate cancer**. *International journal of cancer Journal international du cancer* 2011.

221. Hogervorst JG, Baars BJ, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA: **The carcinogenicity of dietary acrylamide intake: a comparative discussion of epidemiological and experimental animal research**. *Critical reviews in toxicology* 2010, **40**(6):485-512.

# Chapter 2

## Problem and Method

## 2.1. Problem and Method

In 1997, an incident involving tunnel workers in southern Sweden led to a toxicological contamination in which fish died, cows became paralyzed and workers presented reversible mild neurotoxicity [1]. Acrylamide (AA) a component of the grout used to seal the tunnel seemed to be implicated in this toxicological accident, due to its known potential neurotoxicity. However, analysis of samples derived from exposed and non-exposed workers revealed the presence of AA-hemoglobin adducts in non-exposed workers [1]. In addition, and although tobacco smoke can be a possible source of AA background, Bermark [2] has reported that nonsmoker´s samples also contained background levels of the protein adduct of AA. In view of this, the possibility that AA was present in food became a real and worrying consideration. Subsequently, the analysis of a number of foodstuffs were performed and AA was identified has being formed during heat processing of starch-rich foods [3]. According to some authors, AA is formed by the Maillard reduction of the amino acid asparagine with reducing sugars such as fructose or glucose upon heating at temperatures above 120 ºC [4, 5]. This compound is found at relatively high concentrations (μg to mg/Kg) in common food items such as French fries, potato crisps, crisp bread, bread, coffee and biscuits [3, 6, 7]. Based on food contents, the estimated average daily intake of AA in US and Europe through food consumption is about 0.5 μg/Kg body weight, but the intake is often higher in children and adolescents [6]. AA is classified by the International Agency for Research on Cancer [8] as potentially carcinogenic to humans (class 2A) based on animal experiment data. The discovery of AA in many common cooked starchy foods triggered significant challenges to toxicologists, food scientists, national regulatory and public health organizations due to the potential risks of neurotoxicity and cancer. In fact, these findings have raised a great concern in general public health in recent years.

AA is metabolized to glycidamide (GA) in both human and experimental animals. This step seems to be determinant for the formation of several AA-induced DNA adducts observed in rodents [9, 10] as well for the induction of bacterial mutagenicity [11], micronuclei [12] and dominant lethality in mouse spermatids [13].

As abovementioned, AA possesses a range of hazardous properties, being the key effects carcinogenesis, genotoxicity and reproductive toxicity [14]. AA has been shown to cause neurotoxicity in laboratory animals and humans, as well as to induce various types of tumors, including mammary gland [15, 16]. Different epidemiologic studies have reported lack of association between intake of food containing AA and various types of human cancer [17, 18]. However, other epidemiological studies also reported positive associations. In this context, recent reports from Hogervorst *et al.* [17, 19] described an increased risk of ovarian, endometrial and renal cancer in humans with high AA dietary intake. Moreover, Olesen *et al* [20], have recently reported a positive association between AA-hemoglobin adducts concentrations, as a biomarker of AA exposure and estrogen receptor-positive breast cancer. Overall, it is important to highlight that there is a growing concern about the intake of food containing AA and the possible association with several types of cancer.

In view of the importance of AA and GA it is crucial to increase our knowledge on the deleterious effects induced by these agents in mammalian cells. In this context, different biomarkers (exposure, effect and susceptibility) were used in the present study, for the determination of AA and GA genotoxicity and individual susceptibility associated with these chemical compounds. The use of mechanistic biomarkers is important in toxicology and human health. In fact, the relation between different biomarkers is capable to give us valuable insights about the mode of action of these compounds. In this work, chromosomal aberrations (CAs), sister chromatid exchanges (SCEs), comet assay, specific GA-DNA adducts and individual genetic polymorphisms were selected as key biomarkers.

Complementary approaches were used throughout this work. In order to assess genotoxicity of AA and GA-induced, CAs, SCEs and DNA-adducts in V79 Chinese hamster cells, a mammalian cell line essentially devoid of CYP2E1 activity were first evaluated (**Chapter 3**). Later, and with the knowledge that GSH is a key factor for mammalian cell homeostasis, a study of the effect of the GSH modulators, namely evaluation of the effect of buthionine sulfoximine (BSO), an effective inhibitor of GSH synthesis, of GSH-monoethyl ester (GSH-EE), a cell permeable compound that is intracellularly hydrolysed to GSH and also of GSH endogenously added to culture medium in the same cell line were performed using cytotoxicity and clastogenicity endpoints (**Chapter 4**).

With the aim of expanding the previous results obtained in the first chapters to human cells, the quantification of AA and GA-induced SCEs and DNA-adducts in stimulated human lymphocytes was also performed, and the results presented in **Chapter 5**. Moreover, DNA damage accumulation measured by comet assay in whole blood leucocytes from healthy individuals was evaluated and described in **Chapter 6**. Genes encoding the enzymes involved in the metabolism and repair of xenobiotics substances are often polymorphic in humans. Such genetic polymorphisms may result in inter-individual differences in detoxification and clearance of certain chemicals, as well in the repair of certain DNA damage, possibly affecting health-risk assessments. In view of this, the present thesis also addressed the role of individual genetic polymorphisms that can affect metabolism and DNA repair pathways (BER, NER, HRR and NHEJ) on GA-induced genotoxicity assessed by the SCE (**Chapter 5**) and by the alkaline comet assay (**Chapter 6**) in order to evaluate individual susceptibility to GA induced genotoxic effects.

The results obtained in the context of this thesis may help to predict the impact of the genotoxic effects of AA and of its metabolite GA in human health. Moreover, the association of the previously described methods with a food frequency questionnaire can provide valuable information to future epidemiologic studies.

## 2.2. References


1. Hagmar L, Tornqvist M, Nordander C, Rosen I, Bruze M, Kautiainen A, Magnusson AL, Malmberg B, Aprea P, Granath F *et al*: Health effects of occupational exposure to acrylamide using hemoglobin adducts as biomarkers of internal dose. *Scandinavian journal of work, environment & health* 2001, 27(4):219-226.

2. Bergmark E: Hemoglobin adducts of acrylamide and acrylonitrile in laboratory workers, smokers and nonsmokers. *Chemical research in toxicology* 1997, 10(1):78-84.

3. Tareke E, Rydberg P, Karlsson P, Eriksson S, Tornqvist M: Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *Journal of agricultural and food chemistry* 2002, 50(17):4998-5006.

4. Mottram DS, Wedzicha BL, Dodson AT: Acrylamide is formed in the Maillard reaction. *Nature* 2002, 419(6906):448-449.

5. Stadler RH, Blank I, Varga N, Robert F, Hau J, Guy PA, Robert MC, Riediker S: Acrylamide from Maillard reaction products. *Nature* 2002, 419(6906):449-450.

6. Dybing E, Sanner T: Risk assessment of acrylamide in foods. *ToxicolSci* 2003, 75(1):7-15.

7. Konings EJ, Baars AJ, van Klaveren JD, Spanjer MC, Rensen PM, Hiemstra M, van Kooij JA, Peters PW: Acrylamide exposure from foods of the Dutch population and an assessment of the consequent risks. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 2003, 41(11):1569-1579.

8. IARC: Acrylamide. In: *Acrylamide In IARC monographs on the evaluation of carcinogenic risk to humans Volume 60*, edn.: International Agency for Research on Cancer, Lyon; 1994: 389-433.

9. Gamboa da Costa G, Churchwell MI, Hamilton LP, Von Tungeln LS, Beland FA, Marques MM, Doerge DR: DNA adduct formation from acrylamide via conversion to glycidamide in adult and neonatal mice. *Chemical research in toxicology* 2003, 16(10):1328-1337.

10. Segerback D, Calleman CJ, Schroeder JL, Costa LG, Faustman EM: Formation of N-7-(2-carbamoyl-2-hydroxyethyl)guanine in DNA of the mouse and the rat following intraperitoneal administration of [14C]acrylamide. *Carcinogenesis* 1995, 16(5):1161-1165.

11. Parzefall W: Minireview on the toxicity of dietary acrylamide. *Food ChemToxicol* 2008, 46(4):1360-1364.

12. Paulsson B, Grawe J, Tornqvist M: Hemoglobin adducts and micronucleus frequencies in mouse and rat after acrylamide or N-methylolacrylamide treatment. *Mutation research* 2002, 516(1-2):101-111.

13. Ghanayem BI, Witt KL, Kissling GE, Tice RR, Recio L: Absence of acrylamide-induced genotoxicity in CYP2E1-null mice: evidence consistent with a glycidamide-mediated effect. *Mutation research* 2005, 578(1-2):284-297.

14. Carere A: Genotoxicity and carcinogenicity of acrylamide: a critical review. *AnnIstSuperSanita* 2006, 42(2):144-155.

15. Friedman MA, Dulak LH, Stedham MA: A lifetime oncogenicity study in rats with acrylamide. *Fundamental and applied toxicology : official journal of the Society of Toxicology* 1995, 27(1):95-105.

16. Johnson KA, Gorzinski SJ, Bodner KM, Campbell RA, Wolf CH, Friedman MA, Mast RW: Chronic toxicity and oncogenicity study on acrylamide incorporated in the drinking water of Fischer 344 rats. *Toxicology and applied pharmacology* 1986, 85(2):154-168.

17. Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA: Dietary acrylamide intake and the risk of renal cell, bladder, and prostate cancer. *AmJClinNutr* 2008, 87(5):1428-1438.

18. Mucci LA, Wilson KM: Acrylamide intake through diet and human cancer risk. *JAgricFood Chem* 2008, 56(15):6013-6019.

19. Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA: A prospective study of dietary acrylamide intake and the risk of endometrial, ovarian, and breast cancer. *Cancer EpidemiolBiomarkers Prev* 2007, 16(11):2304-2313.

20. Olesen PT, Olsen A, Frandsen H, Frederiksen K, Overvad K, Tjonneland A: Acrylamide exposure and incidence of breast cancer among postmenopausal women in the Danish Diet, Cancer and Health Study. *IntJCancer* 2008, 122(9):2094-2100.

# Chapter 3

## Cytogenetic damage induced by acrylamide and glycidamide in mammalian cells: correlation with specific glycidamide-DNA adducts.

This chapter was adapted from:

"Cytogenetic damage induced by acrylamide and glycidamide in mammalian cells: correlation with specific glycidamide-DNA adducts." Martins C, Oliveira NG, Pingarilho M, Costa GG, Martins V, Marques MM, Beland FA, Churchwell MI, Doerge DR, Rueff J and Gaspar JF. Toxicology Science (2007) 95: 383-90.

**Abstract**

Acrylamide (AA) is a suspected human carcinogen generated in carbohydrate-rich foodstuffs upon heating. Glycidamide (GA), formed via epoxidation, presumably mediated by cytochrome P450 2E1, is thought to be the active metabolite playing a central role in AA genotoxicity. In this work we investigated DNA damage induced by AA and GA in mammalian cells, using V79 Chinese hamster cells. For this purpose, we evaluated two cytogenetic end-points, chromosomal aberrations (CAs) and sister-chromatid exchanges (SCEs), as well as the levels of specific GA-DNA adducts, namely N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua) and N3-(2-carbamoyl-2-hydroxyethyl)adenine (N3-GA-Ade) using high-performance liquid chromatography coupled with tandem mass spectrometry. GA was more cytotoxic and clastogenic than AA. Both AA and GA induced CAs (breaks and gaps) and decreased the mitotic index. GA induced SCEs in a dose-responsive manner; with AA, SCEs were increased at only the highest dose tested (2 mM). A linear dose-response relationship was observed between the GA concentration and the levels of N7-GA-Gua. This adduct was detected for concentrations as low as 1 μM GA. N3-GA-Ade was also detected, but only at very high GA concentrations (≥ 250 μM). There was a very strong correlation between the levels of N7-Gua-GA in the GA- and AA-treated cells and the extent of SCE induction. Such correlation was not apparent for CAs. These data suggest that the induction of SCEs by AA is associated with the metabolism of AA to GA and subsequent formation of depurinating DNA adducts; however, other mechanisms must be involved in the induction of CAs.

## 3.1. Introduction

Acrylamide (AA; Fig. 3.1) is an important industrial chemical that has been produced for about 50 years in Europe, Japan, and the United States. AA has numerous applications; it has been used as starting material for the synthesis of polyacrylamide polymers, which are employed mainly as flocculating agents in water treatment (drinking and waste waters), as flow control agents in oil well operations, in pulp and paper processing, and in mining and mineral processing. AA is also used as an ingredient in several cosmetic formulations and in molecular biology research laboratories [1, 2].

AA was recently found to be generated during the heating of carbohydrate-rich foodstuffs, predominantly from the precursor asparagine [3]. This finding has refocused the interest in this genotoxicant, especially because appreciable amounts of AA are present in Western diets. In fact, some foods (e.g. French fries, potato crisps, bread and breakfast cereals, and coffee) may contain up to 3 ppm of AA [4]. The average daily intake of AA has been estimated at about 0.5-1.0 µg/kg bw in adults and up to twofold higher in 13-year-old children consuming a normal Western diet [4]. Until 2002, AA was mainly regarded as an industrial or occupational toxicant, and the foremost routes of exposure were considered to be dermal absorption and inhalation of aerosols in the workplace; the new data suggest that oral consumption of AA may be a key element for global risk assessment.

In addition to its well-known neurotoxicity [5], the toxicological hazards associated with AA exposure include germ cell mutagenicity [6] and cancer [7] in rodents. AA has been classified as a probable human carcinogen by IARC (group 2A) [1]. This classification is based on experimental rodent models that have shown AA to be carcinogenic, causing tumours at multiple organ sites in both male and female mice and rats, including follicular thyroid tumours, adrenal pheochromocytomas, scrotal mesotheliomas, mammary gland tumours, lung adenomas and carcinomas, glial brain tumours, oral cavity papillomas, and uterine adenocarcinomas (reviewed in [1, 7]). Evidence for the induction of malignant neoplasia by AA in humans is inadequate, mainly because it is quite difficult to associate dietary consumption of AA with a specific cancer outcome. Moreover, occupational studies have failed to show that AA is

carcinogenic to industrial workers. It is therefore extremely important to obtain data on the mechanisms of action of AA, in order to understand how this genotoxicant may affect the human genome.

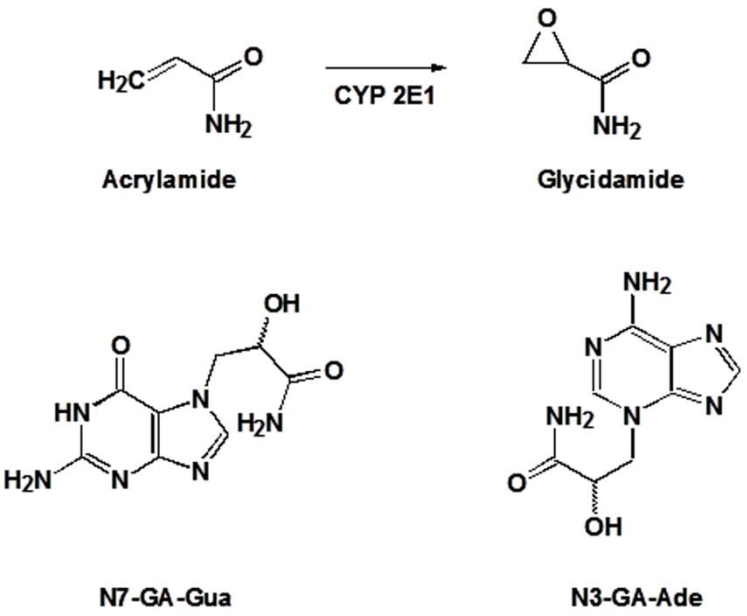



**Fig. 3.1.** CYP2E1-mediated biotransformation of acrylamide to glycidamide and chemical structures of the depurinating adducts (N7-GA-Gua and N3-GA-Ade) mentioned in the text.

AA is metabolized to glycidamide (GA; Fig 3.1) by an epoxidation reaction, presumably mediated by cytochrome P450 (CYP) 2E1 [8-10]. This metabolic conversion appears to be critical for the genotoxicity of AA because when the mutagenicity of AA and GA have been compared, GA has typically been more potent [11-14]. Recently, a number of DNA adducts have been characterized from the interaction of GA with DNA. These adducts include N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua, Fig. 3.1), N3-(2-carbamoyl-2-hydroxyethyl)adenine (N3-GA-Ade; Fig. 3.1), and N1-(2-carboxy-2-hydroxyethyl)-2'-deoxyadenosine (N1-GA-dA) [15, 16]. In this work we have compared the extent of GA-DNA adduct formation induced by AA and GA with the genotoxicity of AA and GA using two different mechanistically based cytogenetic assays: the induction of

chromosomal aberrations (CA) assay and the sister chromatid exchanges (SCE) assay. These assays were performed in V79 Chinese hamster cells, a widely used non-transformed mammalian cell line devoid of cytochrome P450 activity [10, 17].

## 3.2. Materials and methods

### 3.2.1. Chemicals

5-Bromo-2'-deoxyuridine (BrdU), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), trypsin, Ham´s F-10 medium, Hoechst 33258, 30% hydrogen peroxide (w/w), newborn calf serum, mitomycin C, phosphate buffered saline pH 7.4 (PBS), ribonuclease A, and penicillin-streptomycin solution were purchased from Sigma-Aldrich (St. Louis, MO). Dimethylsulphoxide (DMSO), methanol, acetic acid, potassium chloride, sodium chloride, and Giemsa dye were obtained from Merck (Darmstadt, Germany). Colchicine and AA (CAS Registry Number 79-06-1, ≥ 99.5 % pure) were purchased from Fluka (Buchs, Switzerland). GA (CAS Registry Number 5694-00-8, >98.5% pure, containing approximately 1% AA) was obtained from Toronto Research Chemicals (North York, Ontario, Canada).

### 3.2.2. V79 Cells culture

V79 Chinese Hamster cells (MZ), kindly provided by Prof. H.R. Glatt - German Institute of Human Nutrition, Nuthetal, Germany were cultured using Ham's F10 medium, supplemented with 10% newborn calf serum and 1% antibiotic solution (penicillin-streptomycin). The cells growing up at 37ºC in an atmosphere containing 5% $CO_2$.

### 3.2.3. MTT cytotoxicity assay

The MTT assay is based on the reduction of the yellow MTT tetrazolium by mitochondrial desydrogenases to form a blue MTT formazan, in viable cells [18, 19].

Approximately $5 \times 10^3$ V79 in 96-well plates and incubated at 37 ºC under a 5% $CO_2$ atmosphere. The cells were grown for 16 hours and then exposed to different concentrations of AA and GA (dissolved in PBS, pH 7.4), ranging from 100 to 10000 µM, for a 24-h period. Hydrogen peroxide (250 µM) was used as a positive control. The cells were washed with culture medium, incubated with MTT (500 µg/ml) for a further period of 4 h and then carefully washed with PBS. At the end of the incubation period, the media was discarded and DMSO (200 µl) was added to each well. Absorbance was read at 595 nm in a Zenyth 3100 microplate reader. Four independent experiments were performed and eight individual cultures were used for each GA or AA concentration in each independent experiment.

### 3.2.4. Chromosomal aberration assay

Twenty-four hour cultures (approximately 5 x $10^5$ cells), growing in 25 $cm^2$ culture flasks, were exposed to different concentrations of AA and GA, ranging from 1 to 2000 µM, for a period of 16 h. Mitomycin C (750 nM) was used as the positive control. The cells were subsequently washed with fresh culture medium and colchicine was added at a final concentration of 600 ng/ml; the cells were incubated for a further period of 2.5 h and then harvested by trypsinization. After 3-min hypotonic treatment with KCl (0.56%, w/v) at 37 ºC, the cells were fixed with methanol/acetic acid (3:1), and slides were stained with Giemsa 4% [(v/v) in phosphate buffer 0.01 M, pH 6.8] for 10 min, according to Oliveira et al. (2005) [20] and scored [21, 22].

For the quantification of the DNA damage induced by both AA and GA, the index of percentage of aberrant cells excluding gaps (%ACEG) was used. This index represents the frequency of metaphases containing chromosomal aberrations excluding gaps and is the standard indicator for the CA assay. The types of aberrations considered for this index were breaks (chromatid and chromosome), dicentric chromosomes and

rings, chromatid-type rearrangements (triradial, quadriradial), other complex rearrangements, and multi-aberrant cells (MA, cells with more than 10 aberrations, including heavily damaged pulverized cells). The presence of chromatid and chromosome gaps in AA and GA exposed cultures was also evaluated. The index percentage of aberrant cells including gaps (%ACIG) was calculated as mentioned for the %ACEG, including, however, the metaphases containing gaps.

The evaluation of cell proliferation was carried out using the mitotic index (MI). For this index, 1000 V79 cells were scored for each independent experiment and the number of metaphases recorded.

### 3.2.5. Sister chromatid assay

Twenty-four hour cultures (approximately 5 x $10^5$ cells), growing in 25 $cm^2$ culture flasks, were exposed to different concentrations of AA and GA, ranging from 1 to 2000 µM. BrdU was also added at a final concentration of 6 µM. Mitomycin C (750 nM) was used as a positive control. After a period of 27 h, the cells were washed with fresh culture medium and colchicine (600 ng/ml) was added. The cells were then incubated for a further 2.5-h period and then harvested by trypsinization, as described before.

Differential staining of BrdU-substituted sister-chromatids was performed according to the fluorescence-plus-Giemsa (FPG) method [23]. Briefly, the slides were stained for 12 min with the fluorescent dye Hoescht 33258 (10 µg/ml) in 2% KCl (w/v), exposed to UV (254 nm) for approximately 9 min, and then stained with 4% Giemsa [(v/v) in 10 mM phosphate buffer, pH 6.8] for 10 min.

SCEs per cell were scored in 30 second-metaphases for each dose-level in each independent experiment. At least two independent experiments were performed. The evaluation of cell proliferation was carried out using the MI, as described above. At least 100 metaphases per culture for each dose-level, in each independent experiment, were scored for the replication index (RI), calculated according to [24].

### 3.2.6. DNA adducts

#### 3.2.6.1. Chemical exposure and DNA extraction

Twenty-four hour cultures (approximately 8 x $10^6$ cells), growing in 75 $cm^2$ culture flasks, were exposed to different concentrations of AA (0-2000 µM) and GA (0-2000 µM) during two different time periods, 18 h (corresponding to parallel cultures of the CAs assay) and 29 h (corresponding to parallel cultures of the SCE assay). The cells were then harvested by trypsinization as described above, washed with PBS, and the cell suspensions were immediately stored at -20 ºC. DNA was extracted from the cell suspensions using the QIAamp DNA mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, with minor modifications done in order to prevent depurination of the DNA adducts [25]. Cell suspensions (200-250 µl) were lysed with 20 µl of proteinase K, provided by Qiagen, and ribonuclease A (200 µg) for 1 h at 37 ºC and at the end of the chromatographic process DNA samples were eluted in water (200 µl), and stored at -20 ºC for subsequent DNA quantification and determination of DNA adducts.

#### 3.2.6.2. DNA quantification

Quantification of DNA was carried out using a PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR). λ Phage DNA (100 µg/ml) was used as the standard. The DNA concentration in the standard curve ranged from 0 to 300 ng/ml. Briefly, 10 µl of final DNA eluate was mixed with 190 µl of Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) diluted with PicoGreen reagent. Fluorescence intensity was measured in a Zenyth 3100 microplate reader at excitation and emission wavelengths of 485 and 535 nm, respectively. The yield of DNA extracted from each cell suspension was in the range of 20-40 µg, in accordance with the manufaturer's standard yields of DNA for cultured cells.

#### 3.2.6.3. Quantification of DNA adducts

GA-DNA adducts, specifically N7-GA-Gua and N3-GA-Ade (Fig. 3.1), were released from the DNA by neutral thermal hydrolysis and quantified by HPLC coupled with tandem mass spectrometry, essentially as described in Gamboa da Costa et al. [16]. Briefly, aliquots of DNA solutions (~5 µg), containing the $^{15}$N-labeled adducts as internal standards, were heated at 100 ºC for 15 min and then filtered through a prewashed 3-kDa molecular weight cutoff spin filter. The adducts were separated on a 2 x 150 mm C18 analytical column (Luna C18(2), Phenomenex, Torrance, CA) with 2% acetonitrile in water and quantified by tandem mass spectrometry in the multiple reaction monitoring mode, using a Quattro Ultima triple quadrupole mass spectrometer (Waters, Milford, MA) equipped with an electrospray source.

### 3.2.7. Statistical analyses

Dose-related effects were assessed using linear regression analysis. One-way ANOVA, followed by Dunnett's test, was used to compare specific treatment levels to the control group. Two-way ANOVA, followed by Dunnett's test, was used to compare AA and GA treatments. Pearson's Product Moment test was used to assess correlations between variables.

## 3.3. Results

A wide range of AA and GA concentrations were tested in a 24-hour incubation MTT cytotoxicity assay protocol. The average survival values obtained from four independent experiments with V79 cells treated with AA and GA are depicted in Figure 3.2. Previous experiments revealed no changes in the survival frequency of V79 cells using a 3-hour incubation period with both compounds (data not shown). It is clear from Figure 3.2 that both AA and GA induced dose-dependent cell death, as measured by the MTT assay. Moreover, GA was clearly more cytotoxic than AA, causing lower survival

rates at all the equimolar concentrations studied. Very low (< 5%) survival values, occurred at very high concentrations of GA (≥ 4 mM) and AA (10 mM).

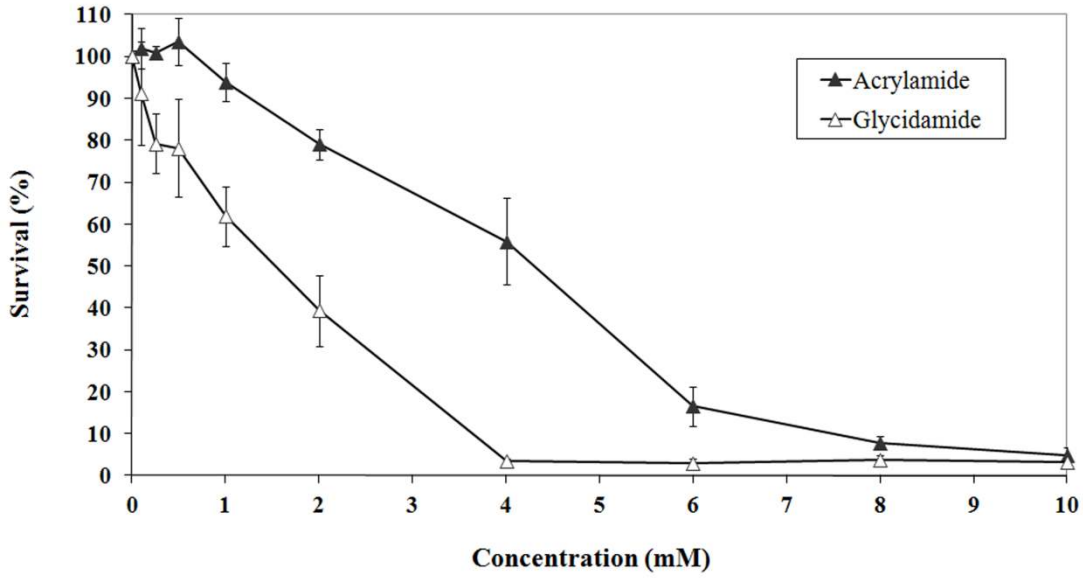



**Fig. 3.2**. Cytotoxicity of acrylamide and glycidamide in V79 cells (MTT assay). The results are expressed as the average ± SEM from four independent experiments.

Both AA and GA induced CAs (Table III.1) and decreased the MI, evaluated as a measure of cell proliferation associated with this cytogenetic end-point. This antiproliferative effect was more pronounced in GA exposure, with the MI being zero at 2000 µM, which prevented the cytogenetic evaluation at this dose level (Table III.1).

AA and its metabolite GA increased the %ACEG, especially for the higher concentrations evaluated (1000 and 2000 µM, for GA and AA, respectively). As observed in the survival assays, GA had, as expected, a more pronounced effect than AA at equimolar concentrations (Table III.1).

**Table III.1**- Induction of chromosomal aberrations in V79 cells by acrylamide and glycidamide.

| Test compound | Chromosomal aberrations per 100 cells[a] | | | | | | MA | Gaps/cell | %ACIG[b] | %ACEG[c] | MI (%)[d] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ctg | Csg | Ctb | Csb | Rearr | Dic+rings | (%) | | | (Average ± S.D.) | (Average ± S.D.) |
| **Acrylamide (μM)** | | | | | | | | | | | |
| **0** | 6.5 | 0.5 | 0.5 | 0 | 0 | 0 | 0 | 0.07 ± 0.03 | 6.0 ± 1.4 | 0.5 ± 0.7 | 9.3 ± 0.1 |
| **250** | 3.5 | 0 | 1.0 | 0 | 0 | 0 | 0 | 0.04 ± 0.02 | 4.5 ± 3.5 | 1.0 ± 1.4 | 8.5 ± 0.4 |
| **500** | 11.5 | 1.5 | 2.0 | 0.5 | 0 | 0 | 0 | 0.13 ± 0.01 | 14.5 ± 3.5 | 2.5 ± 0.7$^{+}$ | 8.4 ± 0.2 |
| **1000** | 17.5 | 0 | 3.0 | 0 | 0 | 0 | 0.5 | 0.18 ± 0.01 | 18.0 ± 4.2 | 3.5 ± 2.1 | 6.9 ± 0.1$^{+}$ |
| **2000** | 19.0 | 1.5 | 8.5 | 1.5 | 0.5 | 0 | 0 | 0.21 ± 0.01 | 27.0 ± 5.7 | 10.0 ± 4.2 | 5.2 ± 1.7$^{+}$ |
| **Glycidamide (μM)** | | | | | | | | | | | |
| **0** | 5.8 | 0.5 | 0.5 | 0 | 0 | 0 | 0 | 0.06 ± 0.02 | 5.5 ± 1.3 | 0.5 ± 0.6 | 9.2 ± 0.1 |
| **1** | 3.0 | 0 | 1.0 | 0 | 0 | 0 | 0 | 0.03 ± 0.01 | 4.0 ± 2.8 | 1.0 ± 1.4 | 8.4 ± 2.0 |
| **2.5** | 6.0 | 0 | 0.5 | 0 | 0.5 | 0 | 0.5 | 0.06 ± 0.03 | 6.5 ± 0.7 | 1.5 ± 0.7 | 8.2 ± 0.1 |
| **5** | 5.5 | 0 | 0.5 | 0 | 0 | 0.5 | 0 | 0.06 ± 0.04 | 6.5 ± 3.5 | 1.0 ± 0.0 | 8.7 ± 0.3 |
| **10** | 4.0 | 0.5 | 1.5 | 0 | 0 | 0 | 0 | 0.05 ± 0.04 | 5.5 ± 3.5 | 1.5 ± 0.7 | 8.0 ± 1.1 |
| **25** | 8.5 | 0.5 | 0.5 | 0 | 0 | 0 | 0.5 | 0.09 ± 0.07 | 9.0 ± 7.1 | 1.0 ± 0.0 | 7.5 ± 0.1 |
| **50** | 7.5 | 0 | 1.5 | 0 | 0 | 0 | 0 | 0.08 ± 0.02 | 8.0 ± 1.4 | 1.5 ± 0.7 | 8.2 ± 1.0 |
| **100** | 10.5 | 0.5 | 1.0 | 0 | 0 | 0 | 0 | 0.11 ± 0.01 | 10.0 ± 1.4 | 1.0 ± 1.4 | 7.2 ± 0.4 |
| **250** | 12.0 | 0.5 | 2.5 | 0 | 0 | 0 | 0 | 0.13 ± 0.02 | 13.5 ± 2.1 | 2.5 ± 0.7$^{*}$ | 8.1 ± 0.6 |
| **500** | 18.5 | 0.5 | 5.5 | 0 | 0.5 | 0 | 0 | 0.19 ± 0.03 | 19.5 ± 0.7 | 6.0 ± 0.0$^{**}$ | 5.8 ± 0.7 |
| **1000** | 29.5 | 1.5 | 6.5 | 0 | 2.0 | 0 | 0.5 | 0.31 ± 0.08 | 30.0 ± 1.4 | 9.0 ± 1.4$^{**}$ | 4.8 ± 0.0 |
| **2000** | - | - | - | - | - | - | - | - | - | - | 0.0 ± 0.0 |
| **Mitomycin C (nM)** | | | | | | | | | | | |
| **750** | 34.3 | 3.7 | 26.0 | 4.3 | 17.7 | 0.3 | 7.0 | 0.38 ± 0.04 | 55.0 ± 1.0 | 40.3 ± 6.7 | 4.4 ± 1.3 |

[a]The results are expressed as the average from two independent experiments (100 metaphases analysed per experiment) for all the points, except for negative V79 cell controls and mitomycin C. In these cases the results are expressed as the average from four independent experiments (100 metaphases analysed per experiment). Ctg, chromatid gap; Csg, chromosome gap; Ctb, chromatid break; Csb, chromosome break; Dic, dicentric chromosome; Rearr, rearrangements (triradial, quadriradial, and other complex rearrangements); [b]%ACIG, percent of aberrant cells including gaps (average ± standard deviation); [c]%ACEG, percent of aberrant cells excluding gaps (average ± standard deviation); MA, multi-aberrant cells, corresponding to cells with more than 10 aberrations. MA are included in the index %ACEG and %ACIG; [d]MI, mitotic index. $^{*}$ $P<0.05$ when compared with control experiment; $^{**}$ $P<0.001$ when compared with control experiment. $^{+}$ $P<0.05$ when compared with the same concentration of glycidamide.

For both AA- and GA-exposed cultures, the CA pattern consisted mainly of chromatid breaks, although some few chromatid-type rearrangements (*e.g.* triradial/quadriradial) were also found for GA. Dicentric and ring chromosomes, and multi-aberrant cells were nearly absent.

Gaps are generally considered to be a minor class of aberrations, and their real biological significance has been a matter of discussion. These events are usually recorded separately from the other aberrations [21, 22]; however, it is clear that both AA and GA are very efficient inducers of chromatid gaps, leading to a consistent dose-response effect ($P<0.001$) (Table III.1). In view of this, we also calculated the %ACIG and the number of gaps per cell (Table III.1). The highest values of %ACIG were about 30% for both the AA-exposed (2000 μM) and GA-exposed (1000 μM) cultures. Moreover, the gaps/cell index revealed a maximum value of 0.2 for AA (2000 μM) and 0.3 for GA (1000 μM) treatment (Table III.1), showing the importance of these aberrations.

Table III.2 presents data on the two cytogenetic indices associated with the SCE assay (SCE/cell and SCE/chromosome), as well as the proliferation indices (mitotic and replicative) associated with exposure to AA and GA. A wide range of GA (1-1000 μM) and AA (250-2000 μM) concentrations were included in this study. The results clearly show that GA consistently induces SCEs for concentrations ≥10 μM (see Table III.2), increasing the background level of SCEs by about 10-fold, to levels of ~60 SCE/cell at the highest concentration tested (1000 μM). For AA-exposed cultures, a significant increase in SCE/cell was only observed for the highest dose tested (Table III.2) and this effect can be considered as mild, since it represents only a ~1.6 fold increase over background.

The levels of N7-GA-Gua and N3-GA-Ade in V79 cell cultures exposed to AA and GA for 18 and 29 hours (corresponding to parallel cultures of the CAs and SCE assays, respectively) are presented in Table III.3. These data show that GA is a potent inducer of N7-GA-Gua, with a linear dose-response dependence ($P<0.001$). For both periods of exposure, the detection of N7-GA-Gua was observed for doses as low as 1 μM GA. In addition, the levels of N7-GA-Gua did not show any significant differences between the two exposure periods tested (Table III.3). In fact, these levels were in the same range for all the concentrations tested, except for the highest concentration of GA

(2000 μM), which showed an approximately twofold increase in the 29-h exposure period when compared to the 18-h period; however, this difference did not reach statistical significance.

**Table III.2**. Sister chromatid exchanges induced by acrylamide and glycidamide in V79 cells

| Test compound | SCE /chromosome[a] (Average ± S.D.[b]) | SCE/metaphase[a] (Average ± S.D.) | MI (%)[c] (Average ± S.D.) | RI[d] (Average ± S.D.) |
|---|---|---|---|---|
| **Acrylamide (μM)** | | | | |
| **0** | 0.31 ± 0.03 | 6.83 ± 0.52 | 4.5 ± 0.4 | 2.00 ± 0.01 |
| **250** | 0.35 ± 0.00 | 7.65 ± 0.02$^{+}$ | 4.3 ± 0.1 | 2.00 ± 0.01 |
| **500** | 0.39 ± 0.01 | 8.33 ± 0.14$^{++}$ | 4.0 ± 0.6 | 2.04 ± 0.06 |
| **1000** | 0.36 ± 0.04 | 7.70 ± 0.90 | 3.9 ± 0.8 | 1.99 ± 0.00 |
| **2000** | 0.51 ± 0.01 | 11.08 ± 0.02$^{*}$ | 4.1 ± 1.0 | 1.99 ± 0.01 |
| **Glycidamide (μM)** | | | | |
| **0** | 0.29 ± 0.02 | 6.19 ± 0.50 | 5.2 ± 0.7 | 1.97 ± 0.04 |
| **1** | 0.33 ± 0.04 | 7.05 ± 0.68 | 6.1 ± 1.3 | 2.08 ± 0.01 |
| **2.5** | 0.35 ± 0.05 | 7.72 ± 1.06 | 5.7 ± 0.6 | 2.06 ± 0.02 |
| **5** | 0.33 ± 0.03 | 7.12 ± 0.68 | 5.5 ± 1.1 | 2.07 ± 0.04 |
| **10** | 0.49 ± 0.13 | 10.53 ± 2.64$^{*}$ | 6.4 ± 0.8 | 2.00 ± 0.03 |
| **25** | 0.54 ± 0.07 | 11.60 ± 1.41$^{**}$ | 4.5 ± 0.2 | 2.04 ± 0.02 |
| **50** | 0.47 ± 0.06 | 10.26 ± 1.38$^{**}$ | 5.1 ± 1.2 | 2.02 ± 0.06 |
| **100** | 0.72 ± 0.07 | 15.42 ± 1.26$^{**}$ | 4.7 ± 1.0 | 2.02 ± 0.02 |
| **250** | 1.10 ± 0.10 | 23.75 ± 2.57$^{**}$ | 4.3 ± 1.1 | 2.00 ± 0.00 |
| **500** | 1.73 ± 0.03 | 37.98 ± 0.21$^{**}$ | 2.8 ± 0.3 | 1.98 ± 0.01 |
| **1000** | 2.71 ± 0.44 | 59.02 ± 9.88$^{**}$ | 1.3 ± 0.5 | 1.96 ± 0.04 |
| **2000** | - | - | 0.0 ± 0.0 | 0.00 ± 0.00 |
| **Mitomycin C (nM)** | | | | |
| **750** | 4.05 ± 0.62 | 88.32 ± 13.70 | 4.4 ± 0.8 | 1.66 ± 0.37 |

[a]The results are expressed as the average from at least two independent experiments (30 metaphases analysed per experiment) for all the points; [b] S.D., standard deviation; [c]MI, mitotic index; [d]RI, replication Index.

$^{*}$ P<0.01 and $^{**}$ P<0.001 when compared with the control experiment.

$^{+}$ P<0.05, $^{++}$ P<0.001 when compared with the same concentration of glycidamide.

N3-GA-Ade was only detected for GA concentrations higher than 250 μM, with a dose-response effect. In addition, the levels of this adduct were in all circumstances two orders of magnitude lower than those of N7-GA-Gua, which is fully consistent with previous data from DNA modifications *in vitro* and from AA and GA administration to

mice [16]. As observed for N7-GA-Gua, the levels of N3-GA-Ade were independent of the exposure time period, although a twofold to threefold decrease was apparent at 29 hours for the highest GA doses tested (1000 and 2000 μM), compared to the 18-h incubation period (Table III.3).

**Table III.3**. Levels of GA-DNA adducts in cell cultures exposed to acrylamide and glycidamide.

| Test compound | 18 hours exposure[a] | | 29 hours exposure[a] | |
|---|---|---|---|---|
| | N7-GA-Gua/ $10^6$ nucleotides | N3-GA-Ade/ $10^6$ nucleotides | N7-GA-Gua/ $10^6$ nucleotides | N3-GA-Ade/ $10^6$ nucleotides |
| **Acrylamide (μM)** | | | | |
| **0** | <LOD[b] | <LOD | <LOD | <LOD |
| **500** | <LOD | <LOD | <LOD | <LOD |
| **1000** | <LOD | <LOD | <LOD | <LOD |
| **2000** | 0.2 ± 0.0 | <LOD | <LOD | <LOD |
| **Glycidamide (μM)** | | | | |
| **0** | <LOD | <LOD | <LOD | <LOD |
| **1** | 0.2 ± 0.0 | <LOD | 0.2 ± 0.0 | <LOD |
| **2.5** | 0.3 ± 0.1 | <LOD | 0.3 ± 0.0 | <LOD |
| **5** | 0.6 ± 0.1 | <LOD | 0.7 ± 0.1 | <LOD |
| **10** | 1.2 ± 0.3 | <LOD | 1.6 ± 0.3 | <LOD |
| **25** | 3.1 ± 0.4 | <LOD | 3.7 ± 1.6 | <LOD |
| **50** | 6.4 ± 0.9 | <LOD | 7.6 ± 2.8 | <LOD |
| **100** | 13.2 ± 2.8 | <LOD | 12.8 ± 1.9 | <LOD |
| **250** | 31.6 ± 3.0 | 0.3 ± 0.4 | 34.2 ± 0.5 | 0.4 ± 0.1 |
| **500** | 50.2 ± 6.2 | 0.9 ± 0.1 | 73.0 ± 9.0 | 1.0 ± 0.2 |
| **1000** | 137.6 ± 69.8 | 2.3 ± 1.3 | 156.8 ± 17.2 | 1.1 ± 0.8 |
| **2000** | 220.4 ± 25.9 | 3.8 ± 1.2 | 488.4 ± 149.4 | 1.3 ± 1.9 |

[a]The results are expressed as the average ± standard deviation from at least two independent experiments; [b]LOD, limit of detection.
The LOD for N7-GA-Gua and N3-GA-Ade, when assaying 5 μg of DNA, was 0.1 adducts/$10^6$ nucleotides.

AA exposure led to very low levels of N7-GA-Gua, which were only observed for concentrations higher than 1000 μM. The adduct levels detected at 2000 μM AA were comparable to those observed for 1 μM GA. N3-GA-Ade was not detected in AA-exposed cultures at any dose level. Negative controls, corresponding to cells not exposed to either AA or GA, did not present any detectable levels of GA-DNA adducts.

The levels of N7-GA-Gua were compared with the levels of cytogenetic damage in AA- and GA-exposed cultures, at the periods of time corresponding to parallel cultures for the SCE and CA assays. A very strong correlation was observed between the levels of N7-GA-Gua and SCE/cell ($r=0.987$; $P=1.25 \times 10^{-12}$).

### 3.4. Discussion

AA is metabolized to GA in mice, rats, and humans [25-27]. The conversion of AA to GA is apparently saturable in rodents [28] and both compounds are detoxified by conjugation with glutathione; in addition, GA can also be detoxified by epoxide hydrolase [1].

The cytotoxic potential of both AA and GA was investigated in this work in order to select the range of concentrations to be tested in the subsequent studies using different cytogenetic end points (CAs, SCEs). The results show that, with our experimental conditions, GA is clearly more cytotoxic than AA for all the concentrations evaluated. Additionally, at concentrations up to 4 mM for AA and up to 1 mM for GA, the cell survival was clearly above 50%, indicating that the cytotoxicity of both compounds would not hinder the cytogenetic studies (Fig. 3.2). These results are in agreement with data recently reported by other groups, using different cell survival end points [11, 12] .

The genotoxicity of AA has been evaluated in several systems (reviewed in [13]). Positive results for the induction of CAs and SCEs in Chinese hamster V79H3 cells at concentrations in the millimolar range were reported by Tsuda *et al*. [29]. These results are in general agreement with the results reported in the present work. Our data showed that AA induced CAs in a dose-response manner, with chromatid gaps and breaks being the typical features observed. However, if we exclude the gap-type aberrations, the genotoxicity observed at the highest AA concentration tested (2 mM), can be considered moderate. Since CYP2E1 activity is not detectable in V79 cells [10,

17], the clastogenicity observed, about 10% ACEG, must be related to mechanisms other than metabolic conversion to GA.

AA may undergo Michael-type additions in particular with thiols, thus potentially depleting the levels of glutathione, a molecule protecting the cell against endogenous oxidants and electrophiles [10]. Michael-type addition reactions, which proceed at very low rates, have also been reported between AA and DNA to yield a series of depurinating and non-depurinating adducts [30]. Additionally, there is some evidence of the involvement of free radicals in AA genotoxicity, leading to oxidative modification of pyrimidines [31]. These mechanisms might, to some extent, explain the clastogenic activity of AA observed in this study. SCEs were induced at only the highest AA dose evaluated (2 mM), which was similar to what was observed for the induction of CAs and also consistent with previously reported data [10, 29]. The same aforementioned reasons for the results of the chromosomal aberrations assay may explain the slight increase in the frequency of SCEs observed in 2 mM AA-treated cultures.

Our results concerning the induction of CAs by GA showed that this compound is approximately twofold more clastogenic than AA and, as observed for AA, chromatid gaps and breaks were the most common features observed (Table III.1). In addition, the induction of chromatid gaps was observed to be dose dependent. Considering that gaps might be a consequence of DNA breaks [32], these data are in agreement with the results obtained by other authors using the comet assay, where GA induced alkali-labile sites [12, 33, 34]. The comparison of the genotoxicity of AA and GA in human lymphoblastoid TK6 cells in three different endpoints (comet assay, micronucleus test and thymidine kinase assay) also suggested that GA is more genotoxic than AA [12], which is in agreement with the data obtained in our cytogenetic end-points.

There are only a few studies comparing, in the same experimental conditions, the genotoxic activity of AA to that of its reactive metabolite, GA. In addition, only limited information is available concerning cytogenetic end-points for both compounds. To our knowledge, this is the first study reporting data from SCEs and CAs for both compounds in the same experimental conditions. Moreover, it should be noted that the serum levels of AA and GA observed in animals exposed to AA are in the same range of concentrations used in this study. In fact, a single oral dose of 50 mg/kg AA in mice

produced peak serum concentrations of AA and GA of approximately 450 and 200 µM, respectively [35] and a repeat dosing through drinking water of approximately 1 mg/kg/day, produced steady state serum concentrations of approximately 500 nM in rats for both AA and GA [36].

The data concerning the levels of N7-GA-Gua after exposure to AA showed that this adduct was only detected for doses higher than 1 mM, but at very low levels (Table IIII.3). Since the V79 cells used in our study are essentially devoid of CYP2E1 activity, the low levels of N7-GA-Gua stemming from AA exposure might be related to either residual metabolism of AA in V79 cells or to a small extent of spontaneous non-enzymatic oxidation to GA, under the aerobic conditions used for the incubations [13].

In cells treated with GA, the measurement of the N7-GA-Gua levels was much more dose-sensitive than the determination of the cytogenetic end-points evaluated in this study. In fact, N7-GA-Gua was detected for concentrations as low as 1 µM GA at two different exposure periods. However, the detection of N3-GA-Ade was only possible for exposure to doses higher than 250 µM, which is consistent with the data previously reported by Gamboa da Costa *et al.* [16]. In that study, the levels of N7-GA-Gua in mice treated with AA were found to be considerably higher than those of N3-GA-Ade, and a similar result was obtained from *in vitro* incubations of GA with DNA. The levels of N7-GA-Gua in V79 cultures, corresponding to parallel cultures of the CAs and SCEs assays (18 and 29 h, respectively), were within the same range. Since the half-life of N7-GA-Gua in DNA was determined to be 42 h at 37 ºC [16] a depurination-related decrease in adduct levels between the 18- and 29-h incubation periods would not be expected to exceed 16%, which is consistent with our observations. Moreover, the absence of a net increase in the levels of N7-GA-Gua at 29 h further suggests that the GA concentrations in the incubation media might be essentially depleted at 18 h, presumably through hydrolysis. Likewise, considering that the half-life of N3-GA-Ade in DNA was estimated to be 14 h at 37 ºC [16], and assuming no mechanisms involved other than spontaneous depurination, a decrease of approximately 42% in the N3-GA-Ade levels would be expected in the 11-h period separating the 18 and 29-h incubations. This is compatible with the apparent decrease in adduct levels observed for N3-GA-Ade at 29 h.

This study shows that GA induced SCEs, in a linear dose-response manner, with a 10-fold increase being observed at 1 mM GA (Table III.2). Moreover, GA was approximately two orders of magnitude more potent than AA, for which SCEs were only induced at the highest dose tested (2 mM). The DNA adducts investigated in the present work are depurinating lesions, known to be formed upon direct reaction of GA with DNA, even at low GA concentrations [16]. The fact that there is an excellent correlation ($r = 0.987$; $P = 1.25 \times 10^{-12}$) between the levels N7-Gua-GA in the GA- and AA-treated cells and the extent of SCE induction strongly suggests that the metabolism of AA to GA and the ensuing formation of depurinating DNA lesions [8, 9] is responsible for the SCE induction.

The repair of the lesions induced by GA was recently associated with the small patch of base excision repair pathway [33]. In addition, the authors also noted that GA is a strong inducer of single strand breaks (SSB). It is well known that base excision repair can lead to the formation of DNA breaks [37]. Likewise, depurination produces abasic sites that can initiate DNA breaks [37]. Therefore, DNA breakage may to some extent explain the higher clastogenic effect of GA, compared with AA.

DNA breaks can also be repaired by homologous recombination. This type of repair is important for double strand break repair in late S and in G2 phases of the cell-cycle [38]. However, in the case of an unrepaired SSB, conversion into a double strand break can occur. This event may take place during replication, collapsing the replication fork and leaving one free DNA end that is a substrate for homologous recombination [39]. This newly created double-strand break may initiate a SCE by homologous recombination after two subsequent mitotic steps. There is growing evidence that SCEs are formed from persisting SSB; for example, cells deficient in SSB repair have increased levels of SCEs [39]. It should be stressed that while only a moderate increase in clastogenicity was observed with GA, compared to AA, there were substantial differences (two to three orders of magnitude) in the levels of N7-GA-Gua between cells treated with equimolar doses of AA and GA. Thus, while there appears to be a causal relationship between depurinating adduct levels and SCEs/cell, other mechanisms must be involved in the induction of the other cytogenetic end points measured in this study. For example, while GA should be intrinsically less reactive than AA with free radicals, due to the absence of the olefinic double bond, it is still a potent electrophile that may contribute to glutathione depletion, thus increasing vulnerability

of the cells to oxidative damage, as suggested for AA [31]. Additionally, nondepurinating GA adducts (*e.g.*, N1-GA-dA), and depurinating and nondepurinating adducts from direct reaction of AA with DNA [30] might play a role in the cytogenetic responses.

In summary, these results are consistent with the conclusion that the induction of SCEs by AA is associated with the metabolism of AA to GA and subsequent formation of depurinating DNA adducts. Other mechanisms, however, must be involved in the formation of CAs. The elucidation of these mechanisms warrants further investigation.

## 3.5. References


1. IARC: **Acrylamide**. In: *Acrylamide In IARC monographs on the evaluation of carcinogenic risk to humans Volume 60*, edn.: International Agency for Research on Cancer, Lyon; 1994: 389-433.

2. Maniere I, Godard T, Doerge DR, Churchwell MI, Guffroy M, Laurentie M, Poul JM: **DNA damage and DNA adduct formation in rat tissues following oral administration of acrylamide**. *Mutation research* 2005, **580**(1-2):119-129.

3. Stadler RH, Blank I, Varga N, Robert F, Hau J, Guy PA, Robert MC, Riediker S: **Acrylamide from Maillard reaction products**. *Nature* 2002, **419**(6906):449-450.

4. Dybing E, Sanner T: **Risk assessment of acrylamide in foods**. *ToxicolSci* 2003, **75**(1):7-15.

5. LoPachin RM: **The changing view of acrylamide neurotoxicity**. *Neurotoxicology* 2004, **25**(4):617-630.

6. Dearfield KL, Douglas GR, Ehling UH, Moore MM, Sega GA, Brusick DJ: **Acrylamide: a review of its genotoxicity and an assessment of heritable genetic risk**. *MutatRes* 1995, **330**(1-2):71-99.

7. Rice JM: **The carcinogenicity of acrylamide**. *MutatRes* 2005, **580**(1-2):3-20.

8. Ghanayem BI, McDaniel LP, Churchwell MI, Twaddle NC, Snyder R, Fennell TR, Doerge DR: **Role of CYP2E1 in the epoxidation of acrylamide to glycidamide and formation of DNA and hemoglobin adducts**. *Toxicological sciences : an official journal of the Society of Toxicology* 2005, **88**(2):311-318.

9. Ghanayem BI, Witt KL, Kissling GE, Tice RR, Recio L: **Absence of acrylamide-induced genotoxicity in CYP2E1-null mice: evidence consistent with a glycidamide-mediated effect**. *Mutation research* 2005, **578**(1-2):284-297.

10. Glatt H, Schneider H, Liu Y: **V79-hCYP2E1-hSULT1A1, a cell line for the sensitive detection of genotoxic effects induced by carbohydrate pyrolysis products and other food-borne chemicals**. *MutatRes* 2005, **580**(1-2):41-52.

11. Baum M, Fauth E, Fritzen S, Herrmann A, Mertes P, Merz K, Rudolphi M, Zankl H, Eisenbrand G: **Acrylamide and glycidamide: genotoxic effects in V79-cells and human blood**. *Mutation research* 2005, **580**(1-2):61-69.

12. Koyama N, Sakamoto H, Sakuraba M, Koizumi T, Takashima Y, Hayashi M, Matsufuji H, Yamagata K, Masuda S, Kinae N *et al*: **Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells**. *Mutation research* 2006, **603**(2):151-158.

13. Besaratinia A, Pfeifer GP: **Genotoxicity of acrylamide and glycidamide**. *JNatlCancer Inst* 2004, **96**(13):1023-1029.

14. Manjanatha MG, Aidoo A, Shelton SD, Bishop ME, McDaniel LP, Lyn-Cook LE, Doerge DR: **Genotoxicity of acrylamide and its metabolite glycidamide administered in drinking water to male and female Big Blue mice**. *Environmental and molecular mutagenesis* 2006, **47**(1):6-17.

15. Segerback D, Calleman CJ, Schroeder JL, Costa LG, Faustman EM: **Formation of N-7-(2-carbamoyl-2-hydroxyethyl)guanine in DNA of the mouse and the rat following intraperitoneal administration of [14C]acrylamide**. *Carcinogenesis* 1995, **16**(5):1161-1165.

16. Gamboa da Costa G, Churchwell MI, Hamilton LP, Von Tungeln LS, Beland FA, Marques MM, Doerge DR: **DNA adduct formation from acrylamide via conversion to glycidamide in adult and neonatal mice**. *Chemical research in toxicology* 2003, **16**(10):1328-1337.

17. Doehmer J, Dogra S, Friedberg T, Monier S, Adesnik M, Glatt H, Oesch F: **Stable expression of rat cytochrome P-450IIB1 cDNA in Chinese hamster cells (V79) and metabolic activation of aflatoxin B1**. *Proceedings of the National Academy of Sciences of the United States of America* 1988, **85**(16):5769-5773.

18. Chiba K, Kawakami K, Tohyama K: **Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells**. *Toxicology in vitro : an international journal published in association with BIBRA* 1998, **12**(3):251-258.

19. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB: **Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing**. *Cancer research* 1987, **47**(4):936-942.

20. Oliveira NG, Castro M, Rodrigues AS, Goncalves IC, Martins C, Toscano Rico JM, Rueff J: **Effect of poly(ADP-ribosyl)ation inhibitors on the genotoxic effects of the boron neutron capture reaction**. *Mutation research* 2005, **583**(1):36-48.

21. Dean BJ, Danford N: **Assays for the detection of chemically-induced chromosome damage in cultured mammalian cells.** In: *Mutagenicity Testing A pratical approach* edn. Edited by Venitt S, Parry JM. Oxford: IRL Press; 1984: 187-232.

22. Swierenga SH, Heddle JA, Sigal EA, Gilman JP, Brillinger RL, Douglas GR, Nestmann ER: **Recommended protocols based on a survey of current practice in genotoxicity testing laboratories, IV. Chromosome aberration and sister-chromatid exchange in Chinese hamster ovary, V79 Chinese hamster lung and human lymphocyte cultures**. *Mutation research* 1991, **246**(2):301-322.

23. Perry P, Wolff S: **New Giemsa method for the differential staining of sister chromatids**. *Nature* 1974, **251**(5471):156-158.

24. Krishna G, Theiss JC: **Concurrent analysis of cytogenetic damage in vivo: a multiple endpoint-multiple tissue approach**. *Environmental and molecular mutagenesis* 1995, **25**(4):314-320.

25. Doerge DR, Young JF, McDaniel LP, Twaddle NC, Churchwell MI: **Toxicokinetics of acrylamide and glycidamide in Fischer 344 rats**. *Toxicology and applied pharmacology* 2005, **208**(3):199-209.

26. Doerge DR, Young JF, McDaniel LP, Twaddle NC, Churchwell MI: **Toxicokinetics of acrylamide and glycidamide in B6C3F1 mice**. *Toxicology and applied pharmacology* 2005, **202**(3):258-267.

27. Boettcher MI, Bolt HM, Drexler H, Angerer J: **Excretion of mercapturic acids of acrylamide and glycidamide in human urine after single oral administration of deuterium-labelled acrylamide**. *Archives of toxicology* 2006, **80**(2):55-61.

28. Bergmark E, Calleman CJ, Costa LG: **Formation of hemoglobin adducts of acrylamide and its epoxide metabolite glycidamide in the rat**. *Toxicology and applied pharmacology* 1991, **111**(2):352-363.

29. Tsuda H, Shimizu CS, Taketomi MK, Hasegawa MM, Hamada A, Kawata KM, Inui N: **Acrylamide; induction of DNA damage, chromosomal aberrations and cell transformation without gene mutations**. *Mutagenesis* 1993, **8**(1):23-29.

30. Solomon JJ, Fedyk J, Mukai F, Segal A: **Direct alkylation of 2'-deoxynucleosides and DNA following in vitro reaction with acrylamide**. *Cancer research* 1985, **45**(8):3465-3470.

31. Blasiak J, Gloc E, Wozniak K, Czechowska A: **Genotoxicity of acrylamide in human lymphocytes**. *Chemico-biological interactions* 2004, **149**(2-3):137-149.

32. Savage JR: **On the nature of visible chromosomal gaps and breaks**. *Cytogenetic and genome research* 2004, **104**(1-4):46-55.

33. Johansson F, Lundell T, Rydberg P, Erixon K, Jenssen D: **Mutagenicity and DNA repair of glycidamide-induced adducts in mammalian cells**. *Mutation research* 2005, **580**(1-2):81-89.

34. Puppel N, Tjaden Z, Fueller F, Marko D: **DNA strand breaking capacity of acrylamide and glycidamide in mammalian cells**. *Mutation research* 2005, **580**(1-2):71-80.

35. Twaddle NC, McDaniel LP, Gamboa da Costa G, Churchwell MI, Beland FA, Doerge DR: **Determination of acrylamide and glycidamide serum toxicokinetics in B6C3F1 mice using LC-ES/MS/MS**. *Cancer letters* 2004, **207**(1):9-17.

36. Doerge DR, da Costa GG, McDaniel LP, Churchwell MI, Twaddle NC, Beland FA: **DNA adducts derived from administration of acrylamide and glycidamide to mice and rats**. *Mutation research* 2005, **580**(1-2):131-141.

37. Friedberg EC, Walker GC, Siede W: **DNA repair and Mutagenesis**. Washington: ASM Press; 1995.

38. Jackson SP: **Sensing and repairing DNA double-strand breaks**. *Carcinogenesis* 2002, **23**(5):687-696.

39. Helleday T: **Pathways for mitotic homologous recombination in mammalian cells**. *Mutation research* 2003, **532**(1-2):103-115.

# Chapter 4

## Cytotoxicity and chromosomal aberrations induced by acrylamide in V79 cells: Role of glutathione modulators.

This chapter was adapted from:

"Cytotoxicity and chromosomal aberrations induced by acrylamide in V79 cells: Role of glutathione modulators." Oliveira NG, Pingarilho M, Martins C, Fernandes AS, Vaz S, Martins V, Rueff J and Gaspar JF. Mutation Research (2009) 676: 87-92.

**Abstract**

Acrylamide (AA) is a suspected human carcinogen found to be generated during the heating of carbohydrate-rich foodstuffs. AA exhibits 'Michael-type' reactivity towards reduced glutathione (GSH), resulting *in vivo* in the urinary excretion of mercapturic acid conjugates. GSH is a key factor for mammalian cell homeostasis, with diverse functions that include, among others, the conjugation of electrophilic compounds and the detoxification of products generated by oxidative stress. Therefore, studies focusing on the modulation of GSH are of great importance for the understanding of the mechanisms of AA induced toxicity. This report addresses this issue by analyzing cytotoxicity (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay) and clastogenicity (chromosomal aberrations) as endpoints in V79 cells exposed to AA. The experiments described herein include the evaluation of the effect of buthionine sulfoximine (BSO), an effective inhibitor of GSH synthesis, GSH-monoethyl ester (GSH-EE), a compound that is taken up by cells and intracellularly hydrolysed to GSH, and also GSH exogenously added to culture medium. Pre-treatment with BSO increased the cytotoxicity and the frequency of aberrant cells excluding gaps (ACEG) induced by AA. While pre-treatment with GSH-EE did not modify the cytotoxicity or the frequency of ACEG induced by AA, co-treatment with AA and GSH decreased both parameters, rendering the cells less prone to the toxic effects of AA. *In vitro* studies in a cell-free system, using monochlorobimane (MCB), a fluorescent probe for GSH, were also performed in order to evaluate the role of AA in GSH depletion. The results obtained showed that spontaneous conjugation of AA with GSH in the extracellular medium is involved in the protection given by GSH. In summary, these results reinforce the role of GSH in the modulation of the cytotoxic and clastogenic effects induced by AA, which may be relevant in an *in vivo* exposure scenario.

## 4.1. Introduction

Acrylamide (AA) is a suspected human carcinogen generated during the heating of carbohydrate-rich foodstuffs, predominantly from the precursor asparagine [1]. Acrylamide toxicity to mammalian cells has been described and characterized in the last few years using different approaches [2, 3].

AA is metabolized to glycidamide (GA) by an epoxidation reaction, presumably mediated by cytochrome P450 2E1 (CYP2E1) [4, 5]. This metabolic conversion appears to be critical for the genotoxicity of AA [2, 3, 6, 7]. Recently, we have also found that AA, compared to GA, is clearly less cytotoxic and genotoxic, as evaluated by different endpoints [8]. In fact, either the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay or the DNA damage endpoints studied, *viz* chromosomal aberrations (CAs), sister-chromatid exchanges (SCEs) and glycidamide-DNA adducts (GA-DNA adducts), showed that GA unequivocally possesses more toxicity than AA. However, while the importance of GA is nowadays convincing, further studies focusing on the metabolic fate of AA are still needed for a better mechanistic understanding of its toxicity. The modulation of reduced glutathione (GSH) status could give additional insight into this matter. GSH has several crucial roles in mammalian cell including, among others, the conjugation of electrophilic compounds and the detoxification of products generated by oxidative stress [9]. Both GSH functions can indeed be important for mitigation of AA toxicity in an *in vivo* exposure scenario. In fact, besides the possibility of AA being an oxidative stress inducer [10-13], this compound is efficiently conjugated with reduced glutathione (GSH) [3, 14]. The α,ß-unsaturated carbonyl group of AA allows its 'Michael-type' reactivity toward GSH, resulting in the urinary excretion of mercapturic acid conjugates [3]. The conjugates of GSH with AA have been quantified in humans and their importance reported. In fact, toxicokinetic studies in humans have shown that ~60% of AA can be recovered in the urine [15], essentially in the form of GSH conjugates [16]. GA is also conjugated with GSH, but the ratio of glycidamide-GSH to acrylamide–GSH conjugates excreted in human urine is only ~0.1 [15].

In this report, we describe the evaluation of the effect of the GSH modulators buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis, and GSH-monoethyl ester (GSH-EE), a compound that is taken up bycells and intracellularly hydrolysed to GSH. While BSO pre-treatment effectively reduces the endogenous content in GSH [17], the pre-loading of mammalian cells with GSH-EE has proven to significantly increase GSH intracellular content [18]. In this work, we performed cell viability studies, using the MTT reduction assay and also evaluated the levels of chromosomal damage by use the chromosomal aberrations assay in V79 Chinese hamster fibroblasts. In addition, the simultaneous treatment of AA with exogenously added GSH was studied using the same endpoints. In this co-treatment protocol the effect of GSH is essentially extracellular, since this compound does not easily enter the cells [19]. In view of this, we have performed *in vitro* studies in a cell-free system, using monochlorobimane (MCB), a fluorescent probe for GSH [20], in order to evaluate the depletion of GSH in the presence of AA and thus the role of the spontaneous, non-enzymatic conjugation of AA with GSH.

## 4.2. Materials and methods

### 4.2.1. Chemicals

L-Glutathione reduced (GSH; CAS registry number 70-18-8), glutathione reduced monoethyl ester (GSH-EE; CAS registry number 92614-59-0), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), trypsin, newborn calf serum, Ham's F-10 medium, phosphate buffered saline pH 7.4 (PBS), mitomycin C, and penicillin-streptomycin solution were purchased from Sigma-Aldrich (St. Louis, MO). Dimethylsulphoxide (DMSO), ethanol, methanol, acetic acid, potassium chloride, sodium chloride, and Giemsa dye were obtained from Merck (Darmstadt, Germany). Colchicine, monochlorobimane (MCB), L-buthionine sulphoximine (BSO; CAS

registry number 83730-53-4), and acrylamide (AA; CAS registry number 79-06-1, ≥ 99.5 % pure) were purchased from Fluka (Buchs, Switzerland).

### 4.2.2. MTT reduction assay

Cytotoxicity assay was carried out in V79 cells, cultured as described in Capter 3 (3.2.2). The MTT assay was performed according to Martins *et al.* [8], as described in Chapter 3 (3.2.3). Ten-hour cultures, growing in 96-well microplates, were incubated with BSO (0.1 mM) or GSH-EE (1.0 and 2.5 mM) for a period of 14 h. Afterwards, the medium was removed, the cells were washed with culture medium, and exposed to AA (2.0 and 4.0 mM) for 24 h. In the co-incubation experiments, cells exposed to AA (0.5-6.0 mM) were simultaneously incubated with GSH (1.0 mM). After 24 h of incubation, the cells were washed and MTT (0.5 mg/ml) was added to each well. The cells were grown for a further period of 3 h and the assay was carried out as described in the previous chapter. Absorbance values presented by V79 cell cultures without the addition of AA, BSO, GSH-EE or GSH, i.e. control cultures, correspond to 100% of cell viability. At least two independent experiments were performed. Eight individual cultures were used in each independent experiment.

### 4.2.3. Chromosomal aberrations assay

The chromosomal aberrations assay was performed as described previously [8, 21] and as described in Chapter 3 (3.2.4). V79 cells (24-h cultures) were exposed to 2.0 mM AA for a period of 16 h. BSO (0.1 mM) and GSH-EE (1.0 mM) were added 14 h before the incubation with AA. In the co-incubation experiments, cells were simultaneously exposed to AA and GSH (1.0 and 3.0 mM). Mitomycin C (0.75 μM) was used as the positive control. After the treatments, the assay was carried out as described in the previous chapter. Two independent experiments were performed except

for the co-treatment of AA with GSH 1.0 mM. In this case four independent experiments were performed.

For each independent experiment, 100 well-spread metaphases were observed using a 1250x magnification on a light microscope. Scoring of the different types of aberrations was performed according to published criteria [22, 23]. For the quantification of the DNA damage induced by AA, the index %ACEG (percent of aberrant cells excluding gaps) was used. This index represents the frequency of metaphases containing chromosomal aberrations excluding gaps and constitutes the standard indicator for the chromosomal aberrations assay. The types of aberrations considered for this index were: breaks (chromatid and chromosome), dicentric chromosomes and rings, chromatid-type rearrangements (triradial, quadriradial), other complex rearrangements and multi-aberrant cells (MA, cells with more than 10 aberrations, including heavily damaged pulverized cells).

### 4.2.4. GSH conjugation assay

The conjugation studies of AA with GSH were carried out in 96-well black microplates using an *in vitro* cell-free assay based on the MCB fluorimetric method [20, 24]. MCB is a probe that reacts with GSH generating an adduct (MCB-GSH conjugate) that can be detected by fluorimetry [20].

To each well was first added PBS, and afterwards AA (up to 6.0 mM, final concentration) and/or GSH (up to 1.0 mM, final concentration). The microplates were placed in an incubator at 37 ºC, protected from the light for a period of 1 h or 24 h. Afterwards, MCB solution (stock solution of 10 mM in absolute ethanol and diluted 10x with PBS) was added to each well at a final concentration of 100 μM. Control experiments were performed with AA and GSH without any incubation. The plates were then placed in an incubator with shaker for 30 min at 37 ºC (100 rpm, protected from light) and then fluorescence was measured in an Anthos Zenyth 3100 multimode detection microplate reader ($\lambda_{exc}$= 405 nm , $\lambda_{em}$ = 465 nm).

The results were expressed either in fluorescence intensity using relative fluorescence units (RFU) after the subtraction of the background fluorescence or normalized in terms of free GSH (%), i.e. the mean value of fluorescence (RFU) observed in GSH alone plus MCB wells corresponded to 100%. For each experimental point four wells were used in each independent experiment. At least two independent experiments were carried out.

### 4.2.5. Statistical analysis

The Kolmogorov-Smirnov test was used to test the normality of continuous variables (% cell viability, %ACEG). For the variables with a normal distribution the homogeneity of the variances was evaluated using the Levene test, and the differences in the mean values of the results observed in cultures treated with AA *versus* AA+GSH modulators were evaluated by the Student t-test. For non normal variables the Mann-Whitney was used. The levels of significance considered were $P<0.05$, and $P<0.01$. All analyses were performed with the SPSS statistical package (version 15, SPSS Inc. Chicago Il., USA).

## 4.3. Results

### 4.3.1. MTT reduction assay

The results from a set of cell-based experiments using the MTT reduction assay are presented in Fig. 4.1. These experiments were performed in order to evaluate the effect of GSH modulators on the viability of V79 cells treated with AA.

Fig. 4.1.A presents the effect of the depletion of intracellular GSH by pre-incubation of V79 cells with BSO. BSO alone slighlty reduced cell viability compared

with that of control cells (Fig. 4.1.A). The viability of AA-treated V79 cells decreased as a function of AA concentration (Fig. 4.1.A). The pre-incubation with 0.1 mM BSO caused an additional decrease in viability of 35% at 4 mM of AA ($P<0.05$).

The effect of the pre-treatment with GSH-EE on the viability of V79 cells exposed to AA is shown in Fig. 4.1.B. In these experiments, two concentrations of GSH-EE were used (1.0 and 2.5 mM). GSH-EE alone at 1.0 mM concentration was not cytotoxic, but the high concentration (2.5 mM) caused a decrease in cell viability of about 17%. The cell viability in AA-treated cultures was not significantly altered in the presence of both 1.0 and 2.5 mM concentrations of GSH-EE.

Cell viability data corresponding to simultaneous treatment of V79 cells with AA and GSH are presented in Fig. 4.1.C. A slight decrease in cell viability was observed for GSH alone (1.0 mM). Co-treatment with AA (0.5-6.0 mM) and GSH (1.0 mM) consistently enhanced the viability of V79 cells when compared with AA treatment alone. In fact, absolute increases in cell viability of approximately 15-20 % were observed for AA concentrations of 2.0 mM ($P<0.05$), 4.0 mM ($P<0.05$) and 6.0 mM ($P<0.01$) in the presence of GSH (Fig. 4.1.C.).

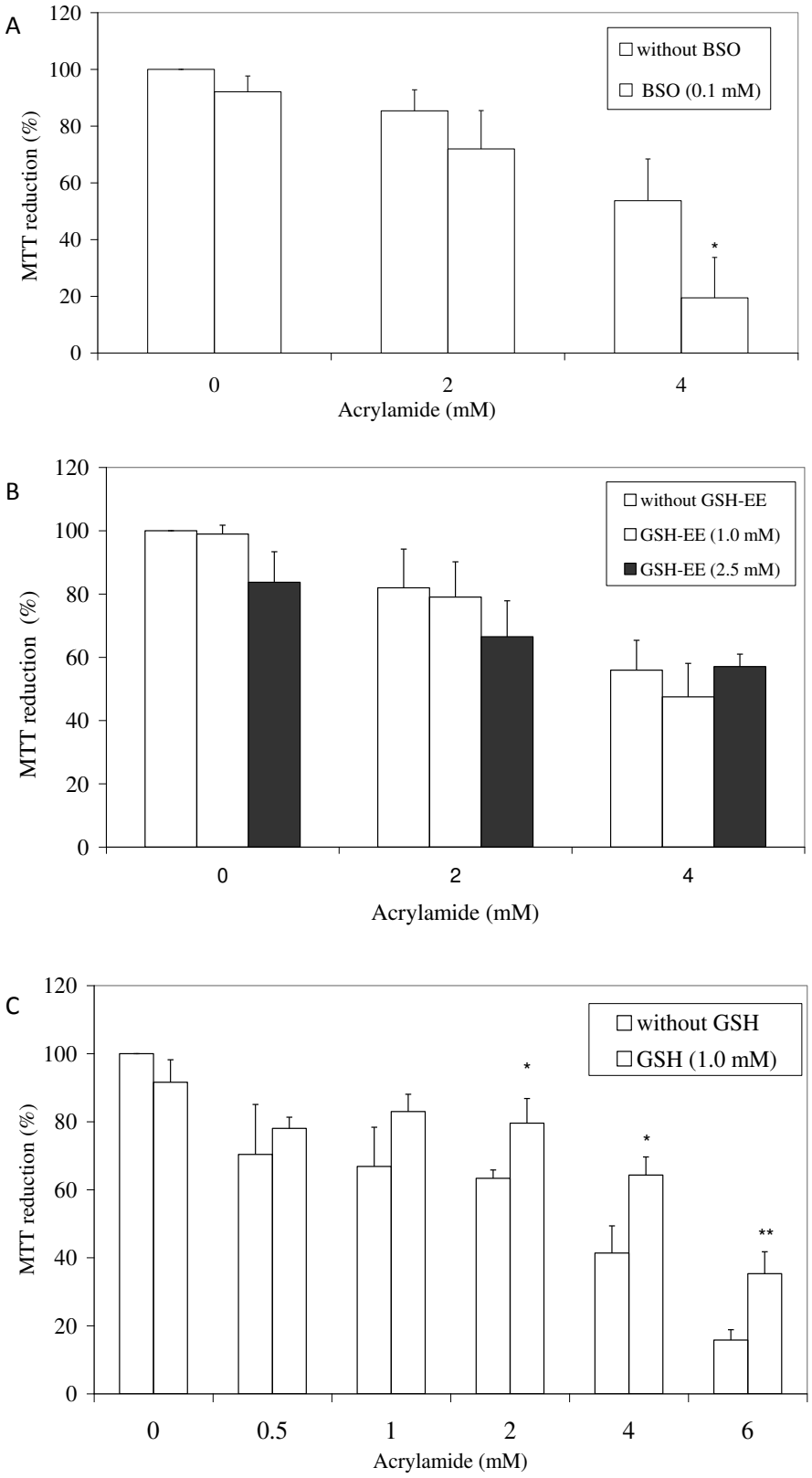



**Fig. 4.1.** Effect of glutathione modulators on cell viability presented by V79 fibroblasts exposed to acrylamide (AA, 24 h incubation) using the MTT reduction assay (% MTT reduction). (A) Effect of BSO (0.1 mM) pre-treatment (14 h). Results are expressed as

mean values and SD from three independent experiments (*P<0.05 when compared with the same concentration of AA in the absence of BSO). (B) Effect of GSH-EE (1.0 and 2.5 mM) pre-treatment (14 h). Results are expressed as mean values and SD from two independent experiments. (C) Effect of GSH (1.0 mM) co-incubation (24 h). Results are expressed as mean values and SD from three independent experiments (*P<0.05 and ** P<0.01 when compared with the same concentration of AA in the absence of GSH).

### 4.3.2. Chromosome aberration assay

The results from the CAs assay in V79 cells exposed to AA are presented in Fig. 4.2. Considering all four independent CA experiments, AA at 2.0 mM significantly increased the frequency of ACEG from ~1% to ~12 % (P<0.05). While pre-treatment with BSO (0.1 mM) caused an increase of the genotoxicity of AA by a factor of 1.5 (not significant), pre-treatment with GSH-EE had no effect on the frequency of ACEG induced by AA (Fig. 4.2.A).

The effect of GSH co-treatment (1.0 mM) on the %ACEG induced by AA is depicted in Fig. 4.2.B. The presence of GSH 1.0 mM led to a decrease of about 25% in the clastogenicity of AA (not significant). GSH 3.0 mM markedly reduced AA-induced ACEG by more than 50%, and this effect almost reached statistical significance (P=0.06). As depicted in Fig. 4.2.B, there is a consistent trend in the decrease of ACEG induced by AA as a function of GSH concentration.

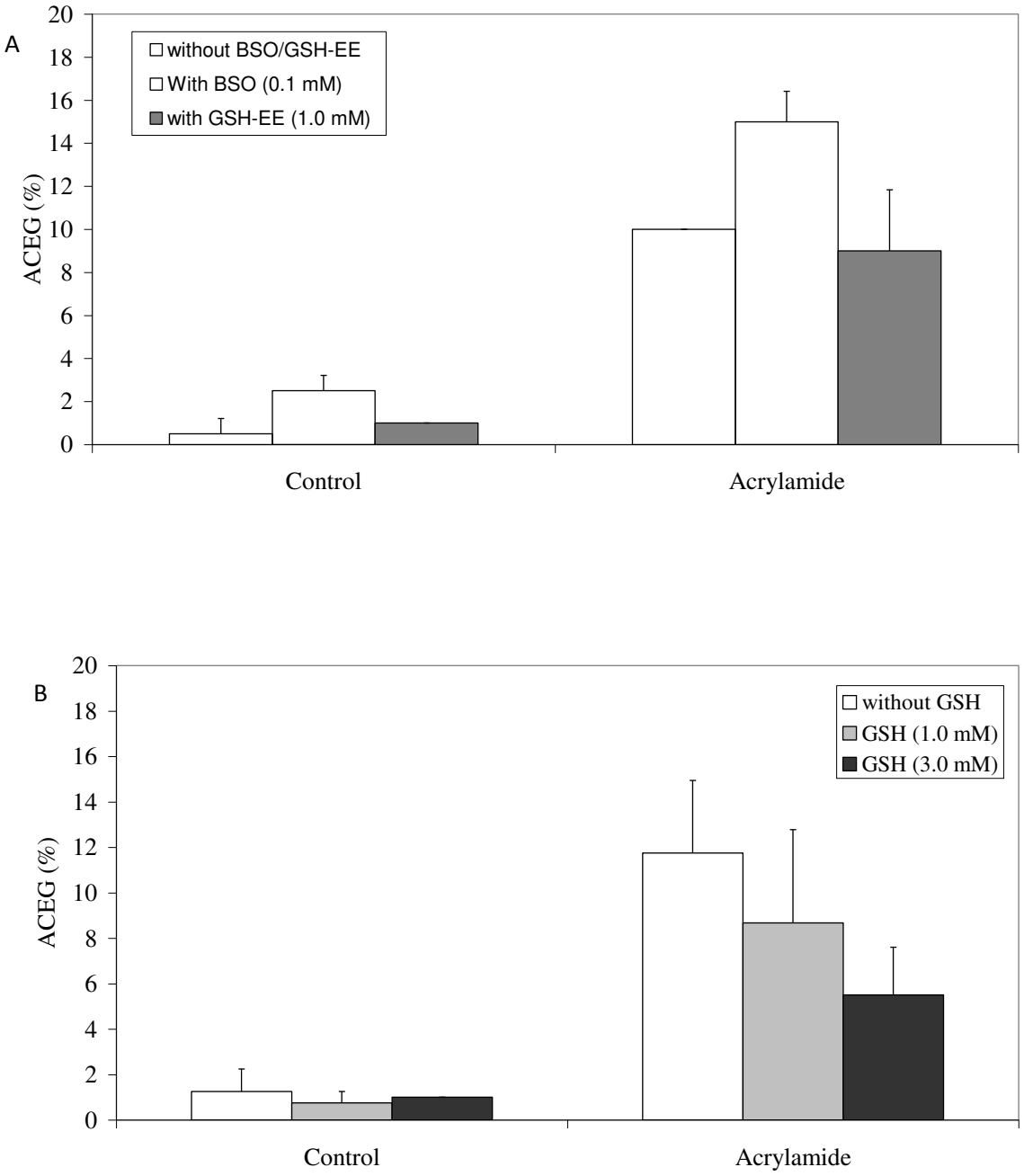



**Fig 4.2.** Effect of glutathione modulators on the induction of chromosomal aberrations by acrylamide (2.0 mM) in V79 cells. (A) Effect of BSO (0.1 mM) or GSH-EE (1.0 mM) pre-treatment (14 h). Results are expressed as mean values and SD from two independent experiments. (B) Effect of GSH (1.0 and 3.0 mM) co-incubation (24 h). Results are expressed as mean values and SD from two (GSH 3.0 mM) or four (GSH 1.0 mM) independent experiments.

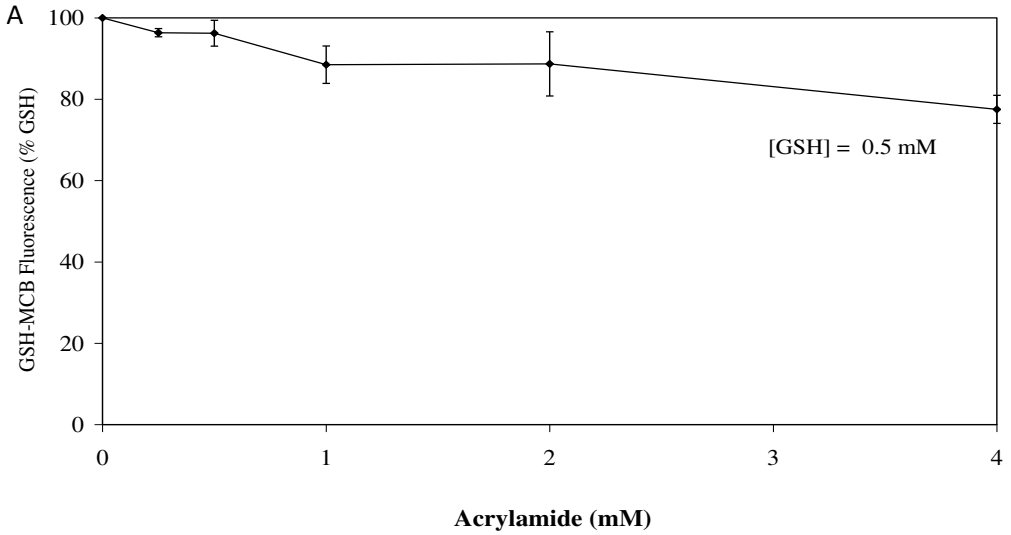
### 4.3.3. GSH conjugation assay

The results from cell-free experiments performed in order to evaluate the spontaneous non-enzymatic conjugation of AA with GSH are presented in Fig. 4.3. Fig. 4.3.A shows the fluorescence curve of the conjugate GSH-MCB for increasing concentrations of GSH, up to 1.0 mM, presenting a clear dose-response and an increase in the intensity of the fluorescence. The pre-treatment by 0.5 mM AA for 1 h (37 ºC) with the same concentrations of GSH did not change the intensity of the fluorescence observed (Fig. 4.3.A).

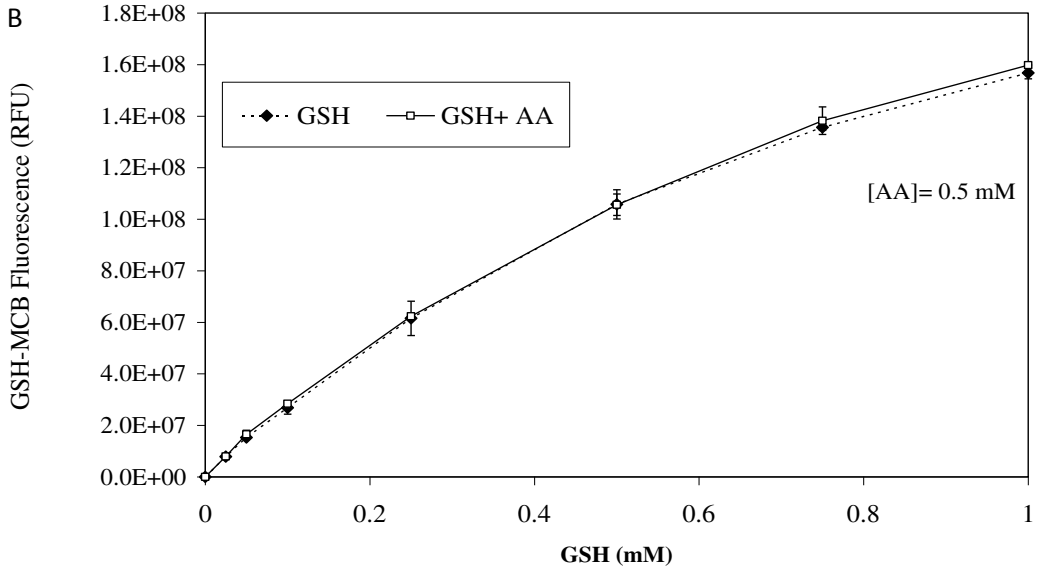
Fig. 4.3.B shows the effect of 1-h pre-treatment with GSH (0.5 mM) with increasing concentrations of AA, up to 4.0 mM. The results expressed in terms of free GSH (%) show a slight conjugation of GSH at different concentrations of AA, which was only evident for the higher concentrations of AA. A control experiment was performed in the same conditions with the purpose of evaluating the immediate conjugation of AA with GSH (without pre-incubation). Under these circumstances no spontaneous conjugation was found (data not shown).

The effect of 24-h pre-treatment of GSH (1.0 mM) with increasing concentrations of AA, up to 6.0 mM is presented in Fig. 4.3.C. The conjugation observed was much more pronounced than for 1-h incubation (Fig. 4.3.B). At the higher concentrations of AA (4.0 and 6.0 mM) the GSH-MCB fluorescence was very low (<3%), which means that almost all the GSH was involved in the conjugation with AA.

A


GSH-MCB Fluorescence (% GSH)
100
80
60
40
20
0
0
1
2
3
4
[GSH] = 0.5 mM
Acrylamide (mM)

B


GSH-MCB Fluorescence (RFU)
1.8E+08
1.6E+08
1.4E+08
1.2E+08
1.0E+08
8.0E+07
6.0E+07
4.0E+07
2.0E+07
0.0E+00
0
0.2
0.4
0.6
0.8
1
GSH
GSH+ AA
[AA]= 0.5 mM
GSH (mM)

C

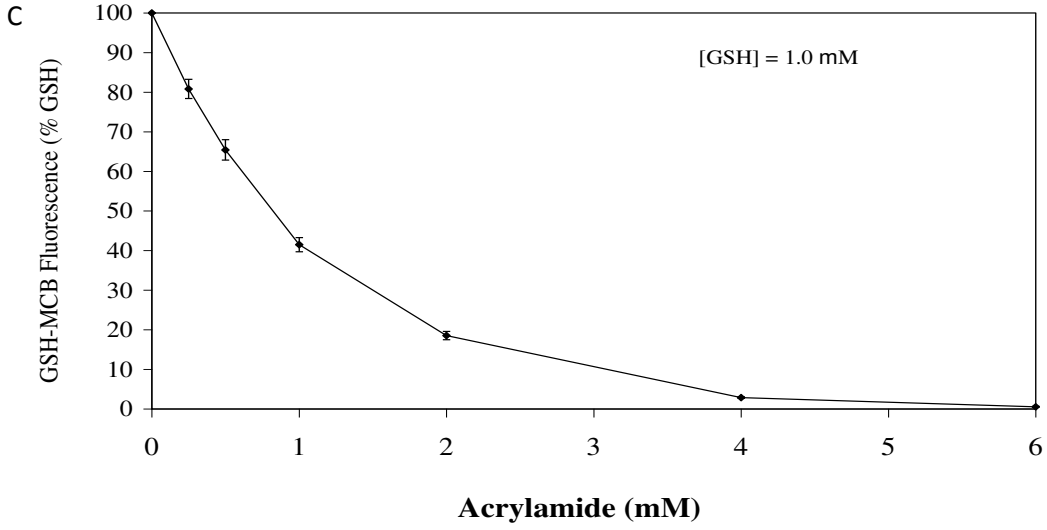

GSH-MCB Fluorescence (% GSH)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
[GSH] = 1.0 mM
Acrylamide (mM)

**Fig. 4.3.** *In vitro* non-enzymatic GSH conjugation studies with acrylamide (AA) using the fluorimetric monochlorobimane (MCB) assay. (A) Dose-effect curves of GSH-MCB fluorescence intensity presented by GSH alone or GSH incubated 1 h with AA (0.5 mM). Results are expressed as mean values and SD of fluorescence intensity (Relative Fluorescence Units -RFU) from two independent experiments. (B) Dose-effect curve of free GSH (%) observed upon incubation of GSH (0.5 mM) during 1 h with different concentrations of AA. Results are normalized in terms of the GSH-MCB fluorescence, considering 100% the fluorescence presented by GSH alone plus MCB. Results are expressed as mean values and SD from three independent experiments. (C) Dose-effect curve of free GSH (%) observed upon incubation of GSH (1.0 mM) during 24 h with different concentrations of AA. Results are normalized in terms of the GSH-MCB fluorescence, considering 100% the fluorescence presented by GSH alone plus MCB. Results are expressed as mean values and SD from two independent experiments.

## 4.4. Discussion

Recently, we have shown that the induction of sister chromatid exchanges and the levels of G7-GA-DNA adducts were found to be two-to-three orders of magnitude lower in AA-treated V79 cells than in GA exposed cells [8]. However, AA cytotoxicity as assessed by the MTT assay and clastogenicity in terms of %ACEG were only about twofold lower when compared with those seen with GA [8]. In view of these clear different effects it seemed important to evaluate the role of GSH modulators in AA-induced toxicity using the aforementioned endpoints of cell viability and clastogenicity in the same cell line. The V79 cells are essentially devoid of CYP2E1 activity [5, 25], being thus adequate for this study, since only negligible amounts of GA are expected to be formed.

In this work, different approaches for the modulation of GSH status were used. First, the effect of endogenous depletion of GSH was studied. Typically, GSH depletion has been carried out by use of BSO, an effective inhibitor of GSH synthesis [17]. In our experimental protocol, V79 cells were incubated with BSO 0.1 mM for a period that approximately corresponds to the duration of one cell cycle (14 h). This concentration of BSO has proven to deplete the GSH content in V79 cells [26]. Other authors using even lower concentrations of BSO also reported marked reductions in the GSH levels in this cell line [27, 28]. In our experiments, BSO treatment alone was associated with

relatively low toxicity leading to a slight decrease in cell viability (Fig. 4.1.A) and to a slight increase in the frequency of ACEG (Fig. 4.2.A).

Several reports have shown the sensitization potential of GSH depletion for the toxicity of physical and chemical agents (reviewed in [29]). As far as AA is concerned, there are few reports focusing on its toxicity in cultured mammalian cells depleted of GSH. Park *et al.* [30] found that BSO increased the percent of morphologically transformed colonies of Syrian hamster embryo cells induced by AA. Recently, other authors described increases in the cytotoxicity of AA in HepG2 cells pre-treated with BSO [12], and others reported enhanced DNA damage as measured by the comet assay in V79 cells pre-treated with BSO [31]. Our results show that the depletion of GSH significantly decreased the viability of AA-treated cells supporting the role of endogenous GSH in the mitigation of the toxic effects triggered by this agent. The clastogenicity of AA was also further enhanced in the presence of BSO. However, this ~1.5 fold increase observed was not significant, which may suggest that, at least in some extent, AA-related cytotoxicity and clastogenicity could be achieved by different mechanisms.

Intracellular GSH enrichment by means of pre-treatment with GSH-EE is a common procedure to evaluate the potential protective effect of GSH towards the toxicity of a given xenobiotic agent. Wellner *et al* [18] showed that the increase in GSH became effective after ~4 h of pre-incubation with GSH-EE and that the GSH content increased either with the duration of the pre-treatment period (up to 24 h) or with the concentration of GSH-EE. In our study, we selected a pre-incubation period of 14 h and two concentrations of GSH-EE, 1.0 mM and 2.5 mM. Longer periods of incubation and higher concentrations of GSH-EE were avoided in order to minimize the possibility of toxicity due to GSH-EE *per se.* In contrast to several reports focusing on the cytoprotective effects of GSH-EE [18, 29, 32-35] there are also some published data where the pre-incubation with GSH-EE did not protect cells against a toxic insult [36-39].

As far as we know there are no previous reports on the effect of GSH-EE against the toxicity induced by AA. The results obtained in this study were consistent with respect to both the cytotoxicity and the clastogenicity endpoint, and fail to show a protective effect of GSH-EE. These results may suggest that the intracellular

concentration of GSH is not the limiting factor for the detoxification of AA in V79 cells, although other issues including the extrusion of GSH-EE as well as some toxicity derived from GSH-EE pre-treatment could be involved. In fact, it has been previously reported that the hydrolysis of the ethyl esters of GSH produces ethanol [18]. This occurrence may be related to the cytotoxicity sometimes associated with GSH monoesters [35, 40], although some authors attribute the toxicity to impurities present in GSH-EE (reviewed in [33]). In this study, we have also observed a modest cytotoxic effect (Fig. 4.1.B) for the higher concentration of GSH-EE (2.5 mM).

While the pre-treatment with GSH-EE readily increases the intracellular concentration of GSH to values significantly above physiological values [32, 40], the incubation with GSH generally leads to small increases in intracellular GSH [32] and therefore can give additional information on extracellular protective effects by GSH. The results presented here show that exogenously added GSH can effectively reduce the cytotoxicity (Fig. 4.1.C) and the induction of chromosomal aberrations (Fig. 4.2.C) by AA. In order to evaluate the effect of a potential conjugation of AA with GSH in the extracellular medium we studied in a set of cell-free experiments the intensity of fluorescence of the conjugate GSH-MCB after incubation of AA with GSH. There are only some reports on the conjugation of AA with GSH in *in vitro* cell-free systems [41]. The experiments herein described with the MCB probe show that the spontaneous conjugation of AA is favoured when AA concentrations are higher than GSH concentrations. Also, these results show that this spontaneous conjugation is clearly time-dependent since 1-h incubation barely decreased the percentage of free GSH when compared to a 24-h incubation period.

The cytotoxicity data from Fig. 4.1.C were obtained in experimental conditions (concentration, incubation period, temperature) comparable to those used in the conjugation studies (Fig. 4.3.C). These results show a marked decrease in free GSH after 24-h incubation, supporting the notion that spontaneous conjugation with AA may be implicated in the reduction of the cytotoxicity of AA when GSH is simultaneously added to the culture medium. However, we should also consider that GSH could be responsible for the scavenging of reactive oxygen species that could be formed by AA in mammalian cells. In fact, some reports have suggested that AA induces oxidative stress in cell-based and animal models [10-13].

It is also interesting to mention that the increase in the viability of V79 cells co-treated with AA and GSH corresponds roughly to 20% in terms of absolute cell viability irrespective of the concentration level. In contrast, the *in vitro* data from the MCB experiments show a clear dose-response decrease in the percentage of free GSH. This apparent lack of correlation may be explained by mechanistic differences between cell-free and cellular systems, since AA is taken up by the cultured cells, and is thus less available for extracellular conjugation with GSH and more prone to exert its toxic effects intracellularly.

In summary, the results presented here reinforce the role of GSH in the modulation of the cytotoxic and clastogenic effects induced by AA. The protection afforded by GSH could be achieved by different mechanisms, including the conjugation with AA and also by an antioxidant-based mechanism. Since GSH is considered to be a key protective factor for mammalian cells and its content may vary at cellular level and also according to patho-physiological conditions, these results may be relevant for an *in vivo* AA exposure scenario.

## 4.5. References


1. Stadler RH, Blank I, Varga N, Robert F, Hau J, Guy PA, Robert MC, Riediker S: **Acrylamide from Maillard reaction products**. *Nature* 2002, **419**(6906):449-450.

2. Parzefall W: **Minireview on the toxicity of dietary acrylamide**. *Food ChemToxicol* 2008, **46**(4):1360-1364.

3. Besaratinia A, Pfeifer GP: **A review of mechanisms of acrylamide carcinogenicity**. *Carcinogenesis* 2007, **28**(3):519-528.

4. Ghanayem BI, Witt KL, Kissling GE, Tice RR, Recio L: **Absence of acrylamide-induced genotoxicity in CYP2E1-null mice: evidence consistent with a glycidamide-mediated effect**. *Mutation research* 2005, **578**(1-2):284-297.

5. Glatt H, Schneider H, Liu Y: **V79-hCYP2E1-hSULT1A1, a cell line for the sensitive detection of genotoxic effects induced by carbohydrate pyrolysis products and other food-borne chemicals**. *MutatRes* 2005, **580**(1-2):41-52.

6. Baum M, Fauth E, Fritzen S, Herrmann A, Mertes P, Merz K, Rudolphi M, Zankl H, Eisenbrand G: **Acrylamide and glycidamide: genotoxic effects in V79-cells and human blood**. *Mutation research* 2005, **580**(1-2):61-69.

7. Manjanatha MG, Aidoo A, Shelton SD, Bishop ME, McDaniel LP, Lyn-Cook LE, Doerge DR: **Genotoxicity of acrylamide and its metabolite glycidamide administered in drinking water to male and female Big Blue mice**. *Environmental and molecular mutagenesis* 2006, **47**(1):6-17.

8. Martins C, Oliveira NG, Pingarilho M, Gamboa da CG, Martins V, Marques MM, Beland FA, Churchwell MI, Doerge DR, Rueff J *et al*: **Cytogenetic damage induced by acrylamide and glycidamide in mammalian cells: correlation with specific glycidamide-DNA adducts**. *ToxicolSci* 2007, **95**(2):383-390.

9. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND: **Glutathione metabolism and its implications for health**. *The Journal of nutrition* 2004, **134**(3):489-492.

10. Blasiak J, Gloc E, Wozniak K, Czechowska A: **Genotoxicity of acrylamide in human lymphocytes**. *Chemico-biological interactions* 2004, **149**(2-3):137-149.

11. Yousef MI, El-Demerdash FM: **Acrylamide-induced oxidative stress and biochemical perturbations in rats**. *Toxicology* 2006, **219**(1-3):133-141.

12. Jiang L, Cao J, An Y, Geng C, Qu S, Zhong L: **Genotoxicity of acrylamide in human hepatoma G2 (HepG2) cells**. *Toxicology in vitro : an international journal published in association with BIBRA* 2007, **21**(8):1486-1492.

13. Zhang X, Jiang L, Geng C, Yoshimura H, Zhong L: **Inhibition of acrylamide genotoxicity in human liver-derived HepG2 cells by the antioxidant hydroxytyrosol**. *Chemico-biological interactions* 2008, **176**(2-3):173-178.

14. Friedman M: **Chemistry, biochemistry, and safety of acrylamide. A review**. *JAgricFood Chem* 2003, **51**(16):4504-4526.

15. Fuhr U, Boettcher MI, Kinzig-Schippers M, Weyer A, Jetter A, Lazar A, Taubert D, Tomalik-Scharte D, Pournara P, Jakob V *et al*: **Toxicokinetics of acrylamide in humans after ingestion of a defined dose in a test meal to improve risk assessment for acrylamide carcinogenicity**. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2006, **15**(2):266-271.

16. Fennell TR, Friedman MA: **Comparison of acrylamide metabolism in humans and rodents**. *Advances in experimental medicine and biology* 2005, **561**:109-116.

17. Dethmers JK, Meister A: **Glutathione export by human lymphoid cells: depletion of glutathione by inhibition of its synthesis decreases export and increases sensitivity to irradiation**. *Proceedings of the National Academy of Sciences of the United States of America* 1981, **78**(12):7492-7496.

18. Wellner VP, Anderson ME, Puri RN, Jensen GL, Meister A: **Radioprotection by glutathione ester: transport of glutathione ester into human lymphoid cells and fibroblasts**. *Proceedings of the National Academy of Sciences of the United States of America* 1984, **81**(15):4732-4735.

19. Halliwell B: **Biochemistry of oxidative stress**. *Biochemical Society transactions* 2007, **35**(Pt 5):1147-1150.

20. Costa-Pereira AP, Cotter TG: **Metabolic alterations associated with apoptosis**. In: *G P Studzinski* edn. Oxford, University Press: Apoptosis. A pratical approach; 1999: 141-156.

21. Oliveira NG, Castro M, Rodrigues AS, Goncalves IC, Martins C, Toscano Rico JM, Rueff J: **Effect of poly(ADP-ribosyl)ation inhibitors on the genotoxic effects of the boron neutron capture reaction**. *Mutation research* 2005, **583**(1):36-48.

22. Dean BJ, Danford N: **Assays for the detection of chemically-induced chromosome damage in cultured mammalian cells.** In: *Mutagenicity Testing A pratical approach* edn. Edited by Venitt S, Parry JM. Oxford: IRL Press; 1984: 187-232.

23. Swierenga SH, Heddle JA, Sigal EA, Gilman JP, Brillinger RL, Douglas GR, Nestmann ER: **Recommended protocols based on a survey of current practice in genotoxicity testing laboratories, IV. Chromosome aberration and sister-chromatid exchange in Chinese hamster ovary, V79 Chinese hamster lung and human lymphocyte cultures**. *Mutation research* 1991, **246**(2):301-322.

24. Debiton E, Madelmont JC, Legault J, Barthomeuf C: **Sanguinarine-induced apoptosis is associated with an early and severe cellular glutathione depletion**. *Cancer chemotherapy and pharmacology* 2003, **51**(6):474-482.

25. Doehmer J, Dogra S, Friedberg T, Monier S, Adesnik M, Glatt H, Oesch F: **Stable expression of rat cytochrome P-450IIB1 cDNA in Chinese hamster cells (V79) and metabolic activation of aflatoxin B1**. *Proceedings of the National Academy of Sciences of the United States of America* 1988, **85**(16):5769-5773.

26. Xue LY, Friedman LR, Oleinick NL: **Repair of chromatin damage in glutathione-depleted V-79 cells: comparison of oxic and hypoxic conditions**. *Radiation research* 1988, **116**(1):89-99.

27. Sugiyama M, Tsuzuki K: **Effect of glutathione depletion on formation of paramagnetic chromium in Chinese hamster V-79 cells**. *FEBS letters* 1994, **341**(2-3):273-276.

28. Grosicka-Maciag E, Kurpios D, Czeczot H, Szumilo M, Skrzycki M, Suchocki P, Rahden-Staron I: **Changes in antioxidant defense systems induced by thiram in V79 Chinese hamster fibroblasts**. *Toxicology in vitro : an international journal published in association with BIBRA* 2008, **22**(1):28-35.

29. Meister A: **Glutathione, ascorbate, and cellular protection**. *Cancer research* 1994, **54**(7 Suppl):1969s-1975s.

30. Park J, Kamendulis LM, Friedman MA, Klaunig JE: **Acrylamide-induced cellular transformation**. *Toxicological sciences : an official journal of the Society of Toxicology* 2002, **65**(2):177-183.

31. Puppel N, Tjaden Z, Fueller F, Marko D: **DNA strand breaking capacity of acrylamide and glycidamide in mammalian cells**. *Mutation research* 2005, **580**(1-2):71-80.

32. Meister A: **Glutathione metabolism and its selective modification**. *The Journal of biological chemistry* 1988, **263**(33):17205-17208.

33. Anderson ME: **Glutathione: an overview of biosynthesis and modulation**. *Chemico-biological interactions* 1998, **111-112**:1-14.

34. Jorquera R, Tanguay RM: **The mutagenicity of the tyrosine metabolite, fumarylacetoacetate, is enhanced by glutathione depletion**. *Biochemical and biophysical research communications* 1997, **232**(1):42-48.

35. Zeevalk GD, Manzino L, Sonsalla PK, Bernard LP: **Characterization of intracellular elevation of glutathione (GSH) with glutathione monoethyl ester and GSH in brain and neuronal cultures: relevance to Parkinson's disease**. *Experimental neurology* 2007, **203**(2):512-520.

36. Spector A, Huang RR, Wang GM, Schmidt C, Yan GZ, Chifflet S: **Does elevated glutathione protect the cell from H2O2 insult?** *Experimental eye research* 1987, **45**(3):453-465.

37. Tsan MF, White JE, Rosano CL: **Modulation of endothelial GSH concentrations: effect of exogenous GSH and GSH monoethyl ester**. *J Appl Physiol* 1989, **66**(3):1029-1034.

38. Gumpricht E, Devereaux MW, Dahl RH, Sokol RJ: **Glutathione status of isolated rat hepatocytes affects bile acid-induced cellular necrosis but not apoptosis**. *Toxicology and applied pharmacology* 2000, **164**(1):102-111.

39. McMillan JM, McMillan DC: **S-adenosylmethionine but not glutathione protects against galactosamine-induced cytotoxicity in rat hepatocyte cultures**. *Toxicology* 2006, **222**(3):175-184.

40. Griffith OW: **Biologic and pharmacologic regulation of mammalian glutathione synthesis**. *Free radical biology & medicine* 1999, **27**(9-10):922-935.

41. Tong GC, Cornwell WK, Means GE: **Reactions of acrylamide with glutathione and serum albumin**. *Toxicology letters* 2004, **147**(2):127-131.

# Chapter 5

## Induction of sister chromatid exchange by acrylamide and glycidamide in human lymphocytes: Role of polymorphisms in detoxification and DNA-repair genes in the genotoxicity of glycidamide

This chapter was adapted from:

"Induction of sister chromatid exchange by acrylamide and glycidamide in human lymphocytes: Role of polymorphisms in detoxification and DNA-repair genes in the genotoxicity of glycidamide." Pingarilho M, Oliveira NG, Martins C, Gomes BC, Fernandes AS, Martins V, Labilloy A, Lima JP, Rueff J, Gaspar JF. Mutation Research (2013), 752, 1-7.

And some data present in this chapter was adapted from:

"Glycidamide-DNA adducts and sister chromatid exchanges in human lymphocytes exposed to acrylamide and glycidamide." 98$^{th}$ Annual Meeting of the American Association for Cancer Research – Los Angeles (Abril 2007). Pingarilho M, Martins C, Oliveira NG, Vaz S, Costa GG, Martins V, Marques MM, Beland FA, Churchwell M, Doerge D, Rueff J and Gaspar JF.

**Abstract**

Acrylamide (AA) is a probable human carcinogen generated in carbohydrate-rich foodstuffs upon heating. Glycidamide (GA), formed via epoxidation, presumably mediated by cytochrome P450 2E1, is considered to be the active metabolite that plays a central role in the genotoxicity of AA. The aim of this work was to evaluate the cytogenetic damage induced by AA and GA in cultured human lymphocytes by use of the sister chromatid exchange (SCE) assay and the levels of specific glycidamide-DNA adducts, namely N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua) and N3-(2-carbamoyl-2-hydroxyethyl)adenine (N3-GA-Ade). Furthermore, this report addresses the role of individual genetic polymorphisms in key genes involved in detoxification and DNA-repair pathways (BER, NER, HRR and NHEJ) on the induction of SCE by GA. While AA induced the number of SCE/metaphase only slightly, especially for the highest concentration tested (2000 μM), GA markedly induced SCEs in a concentration-dependent manner up to concentrations of 750 μM, leading to an increase in SCEs of up to about 10-fold compared with controls. A linear dose-response was observed between the GA concentrations (up to 750 μM) and the level of N7-GA-Gua, with this adduct been detected at the lower studied dose (10 μM). AA-induced DNA adducts were not found at any concentration studied. By combining DNA damage in GA-treated lymphocytes and data on polymorphisms, associations between the induction of SCEs with *GSTP1* (Ile105Val) and *GSTA2* (Glu210Ala) genotypes are suggested.

## 5.1. Introduction

Acrylamide (AA) is a well-known industrial chemical classified as a probable human carcinogen by IARC since 1994 [1]. Until 2002 AA was regarded only as an industrial or occupational genotoxicant. In fact, AA has been used to manufacture polymers, as additives for water treatment, as flow control agent in oil recovery, in pulp and paper processing, in mining and mineral processing and in laboratory gels. The foremost routes of exposure were considered to be dermal absorption and inhalation of aerosols in the workplace [2, 3].

In 2002 it was shown that AA can be formed during heating *via* the Maillard reaction between asparagine and reduced sugars in processed food [4-7]. Moreover, there is evidence that the major contribution for acrylamide-Hb adducts in occupationally non-exposed subjects is originated from acrylamide formation during cooking and food preparation [8]. AA can be found in commonly consumed foods and beverages, such as processed cereals, French fries, potato chips and coffee. Average daily intake of AA was estimated to be about 0.5-1.0 μg/Kg bw in adults and up to 2-fold higher in 13 year-old children with a normal western diet [9].

The metabolism of AA occurs *via* conjugation with reduced glutathione (GSH) resulting in the urinary excretion of a mercapturic acid conjugate, or through epoxidation presumably mediated by cytochrome P450 (CYP2E1) to yiel the genotoxic epoxide glycidamide (GA). GA can be metabolized *via* conjugation with GSH or undergo hydrolysis of the epoxide group by epoxide hydrolase (EPHX) to form glyceramide, which is also excreted in urine [6, 10, 11].

The alpha/ beta-unsaturated double bond of AA is responsible for much of its reactivity, being involved in Michael-type reactions. In fact, the beta-carbon of AA can react with nucleophiles [2], leading to formation of protein adducts (e.g. AA-Hb adducts). In addition, the biological activity of AA is also mediated by its metabolite GA. Besides generating protein adducts, GA has high affinity to DNA, giving rise to DNA-adducts. Conversely, AA has a rather weak capacity to bind DNA [2].

Recently, a number of GA-DNA adducts have been characterized including the N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua, Fig. 5.1), N3-(2-carbamoyl-2-

hydroxyethyl)adenine (N3-GA-Ade, Fig. 5.1), and N1-(2-carboxy-2-hydroxyethyl)-2'-deoxyadenosine (N1-GA-dA) [12].

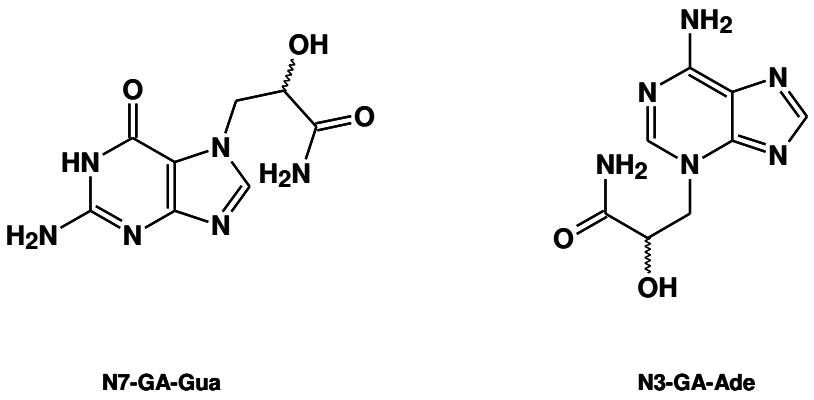



**Figure 5.1 -** Chemical structures of N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua) and N3-(2-carbamoyl-2-hydroxyethyl)adenine (N3-GA-Ade).

Long-term studies in rodent models have shown that AA is carcinogenic at different organ-sites [6]. However, no consistent evidence of an increased cancer risk was found among workers exposed to AA. Moreover, the association of the increased risk for human cancer with dietary consumption of AA is still a matter of discussion [3, 6]. While some studies have found significant associations between oral exposure to AA and cancer, others failed to prove such a relationship. For instance, a recent study by Wilson *et al* [13] found no association between acrylamide and breast cancer. However, with high acrylamide consumption a greater risk for endometrial and possibly ovarian cancer was observed. Similar findings were reported by Hogervorst *et al* [14] whereas Olesen *et al* found a positive association with estrogen-positive breast cancer [15]. In view of the conflicting results in the epidemiological studies it is crucial to develop valuable toxicological biomarkers to be associated with the information in food-frequency questionnaires (FFQ) in order to improve the assessment of carcer risk upon oral consumption of AA.

In this context, the primary aim of the present report is to assess the usefulness of sister chromatid exchange (SCE) as a cytogenetic toxicological biomarker in human lymphocytes exposed *in vitro* to AA and GA and compare this end-point results with the extent of GA-DNA adducts formation. Moreover, this study aims to identify

possible associations of SCE with biomarkers of susceptibility concerning individual genetic polymorphisms in detoxification and DNA-repair genes. The polymorphisms herein studied comprise genes associated with metabolism, specifically glutathione S-transferases (*GSTM1, GSTT1, GSTP1, GSTA2*) and *EPHX1*. Moreover, this study is focused on polymorphisms in DNA-repair genes from the base-excision repair (BER), nucleotide-excision repair (NER), homologous recombination repair (HRR) and non-homologous end-joining repair (NHEJ) pathways, which could be critical in the repair of GA-induced DNA lesions.

## 5.2. Materials and Methods

### 5.2.1. Chemicals

Acrylamide (AA; CAS registry number 79-06-1, ≥99.5% pure) was purchased from Fluka (Buchs, Switzerland) and glycidamide (CAS Registry Number 5694-00-8, >98.5% pure, containing ~1% AA) was obtained from Toronto Research Chemicals (North York, Ontario, Canada). Fetal calf serum, Ham's F-10 medium, penicillin-streptomycin solution, L-glutamine, phosphate-buffered saline pH 7.4 (PBS), methanol, acetic acid, Hoechst 33258, 5-bromo-2'-deoxyuridine (BrdU), mitomycin C, ethidium bromide and colchicine were purchased from Sigma–Aldrich (St. Louis, MO, USA). Potassium chloride and Giemsa dye was acquired from Merck (Darmstadt, Germany). Phytohemagglutinin (PHA) was purchased from Gibco (Grand Island, NY, USA) and heparin was acquired to B. Braun (Lisbon, Portugal).

### 5.2.2. Blood samples collection

Peripheral blood samples were obtained from 13 healthy donors (8 female and 5 male, mean ages 28.1 ± 4.3). The samples were collected under sterile conditions by venipuncture in heparinized tubes, coded and analyzed under blind conditions.

All donors were informed about the aim and experimental details of the study and an informed consent was obtained from all participating subjects prior to the start of the study. Each participant completed one standardized questionnaire about health history, lifestyle, alcohol consumption, medication usage, family history of cancer, exposure to indoor/outdoor pollutants, and dietary habits. All individuals were all non-smokers. Ethical approval for this study was obtained from the institutional Ethical Board of the Faculty of Medical Sciences.

### 5.2.3. Lymphocytes culture

The lymphocytes cultures were set up by adding 0.5 ml of whole blood to 4.5 ml of Ham's F-10 medium supplemented with 24 % fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 1 % L-glutamine, and 50 IU/ml heparin. Lymphocytes were stimulated with 1.5 % (v/v) of PHA and incubated at 37 ºC for 72 h in an atmosphere containing 5 % $CO_2$.

### 5.2.4. Sister chromatid exchange assay

Firstly, different concentrations of AA and GA up to 2000 µM (dissolved in PBS, pH 7.4) were evaluated in order to characterize the dose-response pattern of both chemicals. For this purpose 24-h cultures of lymphocytes from two donors were treated with AA or GA and two independent experiments were performed for each donor. From the dose-response curve of GA, the concentration of 250 µM was chosen to be further assayed in all the donors (two replicate cultures).

For both studies, after 46 h incubation with AA or GA in the presence of BrdU (final concentration of 10 µM), lymphocytes were washed with fresh culture medium and colchicine (0.6 µg/ml) was added. The lymphocytes were then incubated for a further 1.5h. Differential staining of BrdU-substituted sister chromatids was performed according to the fluorescence-plus-Giemsa method [16], as described in Chapter 3

(3.2.5). The frequency of SCE in each metaphase (SCE/metaphase) was scored in 30 second-division metaphases, whenever possible, for each concentration in each experiment. Mitomycin C (0.75 μM) was used as positive control.

### 5.2.5. Mitotic index

The mitotic index (MI) was carried out as a measure of the cell proliferation. This index can be defined as MI = (no. of cells in division / total no. of cells) x 100 [17]. To determine this index, 1000 lymphocytes were scored for each experiment and the number of metaphases recorded.

### 5.2.6. DNA adducts

#### 5.2.6.1. Chemical exposure and DNA extraction.

Twenty-four hour cultures growing in culture tubes, were exposed to different concentrations of AA (0–1000 μM) and GA (0–2000 μM) during 22 hour period (corresponding to parallel cultures of the SCE assay, already explained in Chapter 5.2.4). The lymphocytes were washed with PBS, and the cell suspensions were immediately stored at 20 ºC. DNA was extracted as described in Chapter 3 (3.2.6.1).

### 5.2.7. DNA quantification.

Quantification of DNA was carried out using a Pico-Green dsDNA quantitation kit as described in Chapter 3 (3.2.6.2). The yield of DNA extracted from each cell suspension was in the range of 20–40 μg, in accordance with the manufacturer's standard yields of DNA for lymphocytes cells.

### 5.2.8. Quantification of DNA adducts.

GA-DNA adducts, specifically N7-GA-Gua and N3-GA-Ade, were released from the DNA by neutral thermal hydrolysis and quantified by high-performance liquid chromatography coupled with tandem mass spectrometry, essentially as described in Gamboa da Costa et al [12] as described in Chapter 3 (3.2.6.3).

### 5.2.9. Genotyping

#### 5.2.9.1. Detoxification pathways

Genotyping of *GSTM1* and *GSTT1* for gene deletions were carried out by a multiplex PCR as described by Lin *et al.* [18] with minor modifications described in Costa *et al* [19]. After electrophoretic separation the amplified products were visualized in 2 % agarose gel stained with ethidium bromide (2.0 μg/ml).

The genotyping of *GSTP1* Ile105Val (rs1695), *EPHX1* Tyr113His (rs1051740) and His139Arg (rs2234922) and *GSTA2* Glu210Ala (rs6577) was conducted with the polymerase chain reaction and restriction fragment length polymorphisms (PCR-RFLP). *GSTP1* Ile105Val genotyping was performed according to Gaspar *et al* [20], *EPHX1* Tyr113His and His139Arg polymorphisms were determined as described by Teixeira *et al* [21] and *GSTA2* Glu210Ala polymorphisms were determined according to published procedures [22, 23] with minor modifications. For the *EPHX1* genotypes, in codons 113 and 139, individuals were classified according to the expected activity on the basis of their genotypes as: low activity: His/His–His/His; His/His–His/Arg; Tyr/His–His/His; His/His–Arg/Arg; medium activity: Tyr/Tyr–His/His; Tyr/His–His/Arg; Tyr/His–Arg/Arg; high activity: Tyr/Tyr–Arg/Arg; Tyr/Tyr–His/Arg [22, 24].

#### 5.2.9.2. DNA repair Pathways

The genotyping of *XRCC2* Arg188His (rs3218536), *XRCC3* Thr241Met (rs861539) was performed by means of PCR-RFLP according to Bastos *et al* [25]. *XPC* Lys939Gln (rs2228001) and Ala499Val (rs2228000) polymorphisms were also conducted by means of PCR-RFLP and the primers sequences and PCR product for the polymorphic sites of these genes are shown in Table V.1. For these polymorphisms the nucleotide change resulted in either gain or loss of a restriction site, which therefore allowed the common and variant alleles to be discriminated by RFLP after appropriate enzyme digestion (see Table V.2).

*APEX* Asp148Glu (rs1130409; C___8921503_10), *ERCC1* Gln504Lys (rs3212986; C__2532948_10), *ERCC2* Lys751Gln (rs13181; C___3145033_10), *ERCC4* Arg415Gln (rs1800067; C___3285104_10), *ERCC5* Cys529Ser (rs2227869; C___15956775_10) and His1104Asp (rs17655; C___1891743_10), *ERCC6* Gln1413Arg (rs2228529; C__16171343_10) and Arg1230Pro (rs4253211; C__25762749_10), *GSTA2* Pro110Ser (rs2234951; C__12027714_50) and Ser112Thr (rs2180314; C__22275149_30), *Ku80* Ex21-238G→A (rs2440; C___3231046_20); Ex21+338T→C (rs1051677; C___8838367_1_), Ex21-352C→A (rs6941; C__8838374_10), Ex21+466A→G (rs1051685; C___8838368_1_), *LIG4* Thr9Ile (rs1805388; C__11427969_20), *MUTYH* Gln335His (rs3219489; C__27504565), *NBS1* Glu185Gln (rs1805794; C__26470398_10), *OGG1* Ser326Cys (rs1052133; C___3095552_1_), *PARP1* Val762Ala (rs1136410; C___1515368_1_), *PARP4* Gly1280Arg (rs13428; C___8700143_10) and Pro1328Thr (rs1050112; C___8700142_10), *RAD23B* Ala249Val (rs1805329; C__11493966_10), *RAD51* 5'UTR (rs1801321; C__7482700_10), *XRCC1* Arg194Trp (rs1799782; C__11463404_10) and Gln399Arg (rs25487; C____622564_10) and *XRCC4* It7G>A (rs1805377; C__11685997_10) and Thr134Ile (rs28360135; C25618660_10) polymorphisms were genotyped by Real-Time PCR (AB7300), using TaqMan SNP Genotyping Assays from Applied Biosystems, according to the manufacturer's instructions and previous studies from our group [23, 25-27]. DNA samples were quantified with PicoGreen dsDNA Quantification Reagent (Molecular Probes, Eugene, Ore., USA) according to the manufacturer's recommendations. The SNP genotyping assay information for BER and NER polymorphisms is summarized in Table V.3.

Genotype determinations were carried out twice (all samples for multiplex and PCR-RFLP and 20 % of samples for Real-Time PCR) in independent experiments and all the inconclusive samples were reanalyzed.

### 5.2.10. Statistical Analysis

For the concentration-response curves of AA and GA, regression analyses were performed using the Graphpad Prism v.5 software. The non-parametric Mann-Whitney (for 2 groups comparisons) and Kruskal-Wallis (for more than 2 groups comparisons) tests were used to evaluated the association of different genotypes with the frequency of GA-induced SCE after subtracting the background values. The level of significance considered was $p \leq 0.05$. All analyses were performed with the SPSS statistical package (version 17, SPSS Inc., Chicago, IL, USA).

**Table V.1.** Primer sequences, melting temperature (Tm), GC content and PCR product for *XPC* polymorphisms.

| Primer Sequences | Tm | GC content | PCR product (bp) |
|---|---|---|---|
| ***XPC* Lys939Gln** | | | |
| **Forward** 5´- ACC AGC TCT CAA GCA GAA GC- 3' | 58º C | 55% | 281 |
| **Reverse** 5' -CTG CCT CAG TTT GCC TTC TC- 3´ | 56º C | 55% | |
| ***XPC* Ala499Val** | | | |
| **Forward** 5´- TAA GGA CCC AAG CTT GCC CG- 3' | 51º C | 60% | 152 |
| **Reverse** 5' –CCC ACT TTT CCT CCT GCT CAC AG - 3´ | 52º C | 56% | |

**Table V.2.** – Restriction enzymes used to digest the different PCR products and the respectively digestion time, temperature and restriction patterns for *XPC* polymorphisms.

| Polymorphism and effect on restriction enzyme site | Digestion time and temperature | Restriction patterns after enzyme digestion |
|---|---|---|
| ***XPC* Lys939Gln** | | |
| C→A, create one *Pvu* II site | 2 h 37ºC | **Lys/Lys** : 281 bp<br>**Lys/Gln** : 281 bp + 150 bp + 131 bp<br>**Gln/Gln** : 150 bp +131 bp |
| ***XPC* Ala499Val** | | |
| C→T, create one *Sac* II site | 16-18 h 37ºC | **Ala/Ala**: 152 bp<br>**Ala/Val**: 152 bp + 131 bp + 21 bp<br>**Val/Val**: 131 bp + 21 bp |

**Table V.3.** Single Nucleotide Polymorphisms genotyping assay information for BER and NER pathways.

| Repair Pathway | Gene | SNP region | Amino Acid Exchange | dbSNP ID | ABI ID |
|---|---|---|---|---|---|
| **BER** | *APEX* | | Asp148Glu | rs1130409 | C__8921503_10 |
| | OGG1 | Ex6-315C>G | Ser326Cys | rs1052133 | C___3095552_1_ |
| | *PARP1* | Ex17+8T>C | Val762Ala | rs1136410 | C__1515368_1 |
| | *PARP4* | Ex31+172G>C | Gly1280Arg | rs13428 | C__8700143_10 |
| | | Ex31+316C>A | Pro1328Thr | rs1050112 | C__8700142_10 |
| | *XRCC1* | Ex10-4A>G | Gln399Arg | rs25487 | C____622564_10 |
| **NER** | *ERCC2* | Ex23+61A>C | Lys751Gln | rs13181 | C___3145033_10 |
| | *ERCC5* | Ex15-344G>C | His1104Asp | rs17655 | C___1891743_10 |
| | *ERCC6* | Ex21+176A>G | Gln1413Arg | rs2228529 | C__16171343_10 |
| | | Ex18-90G>C | Arg1230Pro | rs4253211 | C__25762749_10 |
| | *RAD23B* | Ex7+65C>T | Ala249Val | rs1805329 | C__11493966_10 |

## 5.3. Results

### 5.3.1. GA markedly increases the formation of SCE in human lymphocytes

The cytogenetic response in terms of SCE was firstly evaluated in stimulated lymphocytes from whole blood cultures of two healthy individuals. Different concentrations of AA and GA (up to 2000 µM) were used. The individual concentration-response curves for SCE/metaphase obtained for both compounds are presented in Fig. 5.2. The MI was evaluated as a measure of cell proliferation associated with the cytogenetic end-point and both AA and GA decreased the MI. However, the anti-proliferative effect was more pronounced for GA (data not shown). For the concentration of 750 µM, GA decreased the MI to about 29 % of the MI observed in non-treated controls, and a MI of zero was found for 2000 µM. This cytotoxic effect precludes the assessment of SCE frequency for GA concentrations ≥ 1000 µM. For AA, a smaller anti-proliferative effect was noted. However, at a very high concentration of AA (2000 µM) the MI decreased to ~13% of controls (data not shown).

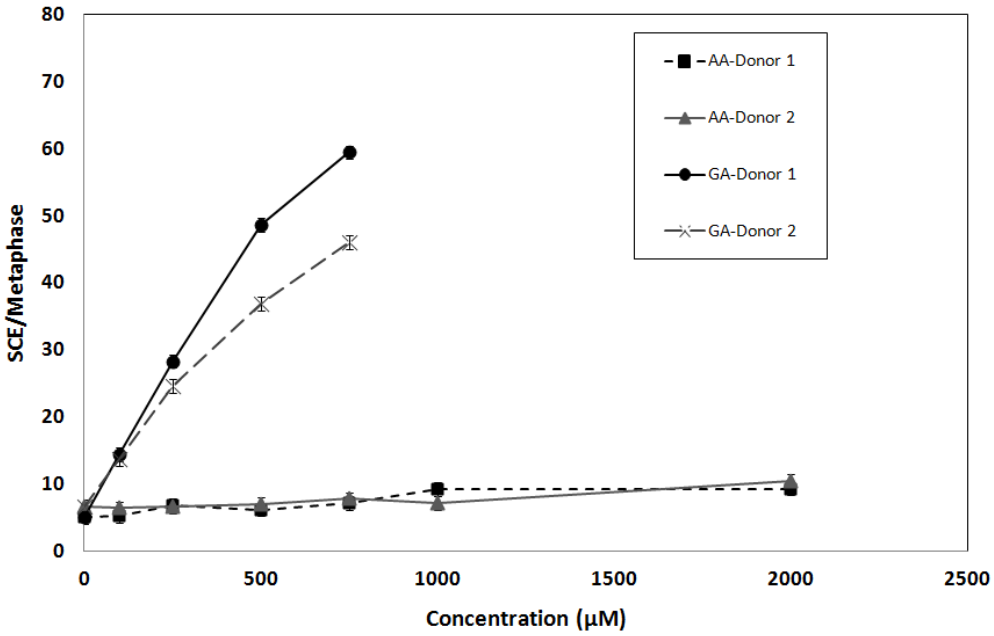



**Fig. 5.2.** Sister chromatid exchanges (SCE/metaphase) induced by acrylamide (AA) and glycidamide (GA) in cultured lymphocytes from two donors. Results are expressed as the mean values ± SD from two independent experiments.

With regards to the induction of SCE, regression analyses performed on the concentration-response curves indicated slopes that were significantly different from zero for AA (slope 0.0022, donor 1; 0.0019 donor 2; $p<0.01$) and GA (slope 0.074, donor 1; 0.052, donor 2; $p<0.01$). These results suggest that AA causes a concentration-dependent increase in SCE, although with a much lower response compared to GA. In fact, AA increased the levels of SCE/metaphase by about 1.5-1.8 fold. This slight increase was particularly observed for the high concentration tested (2000 µM). Conversely, GA was far more genotoxic than AA at all concentrations tested (up to 750 µM), increasing the level of SCE in about 10-fold when compared with control cultures.

### 5.3.2. GA-induced SCE show inter-individual variability

Based on the concentration-response curve of GA (Fig. 5.2), the concentration of 250 µM was selected to be used for the SCE assay on samples from all 13 individuals. This concentration was chosen since it led to a clear genotoxic response of approximately 4.5-fold when compared with non-treated control lymphocytes. Moreover, this concentration caused a decrease of the MI to ~ 62 % of that observed in non-treated controls, which is acceptable for evaluation of chemically-induced chromosome damage [17]. The effect of 250 µM of GA (46 h-exposure) for each donor in terms of SCE/metaphase is depicted in Fig. 5.3 (A). The collective average values and respective standard deviations (SD) for all donors are presented in Figure 5.3 (B). The mean level of SCE/metaphase obtained for the lymphocytes of all individuals whose blood was exposed *in vitro* to 250 µM of GA was 27.2 ± 3.8 while this was 6.6 ± 1.1 for the controls. This represents a significant 4.1-fold increase in number of SCE/metaphase after treatment with GA, compared with the control ($p<0.001$). The results obtained for blood from individual donors treated with GA (Fig 5.3.A) show the extent of inter-individual variability in terms of SCEs results. In fact, the lymphocytes from donors 2, 5 and 9 clearly responded to GA insult to a lesser extent than did donor 6, 8 and 10.

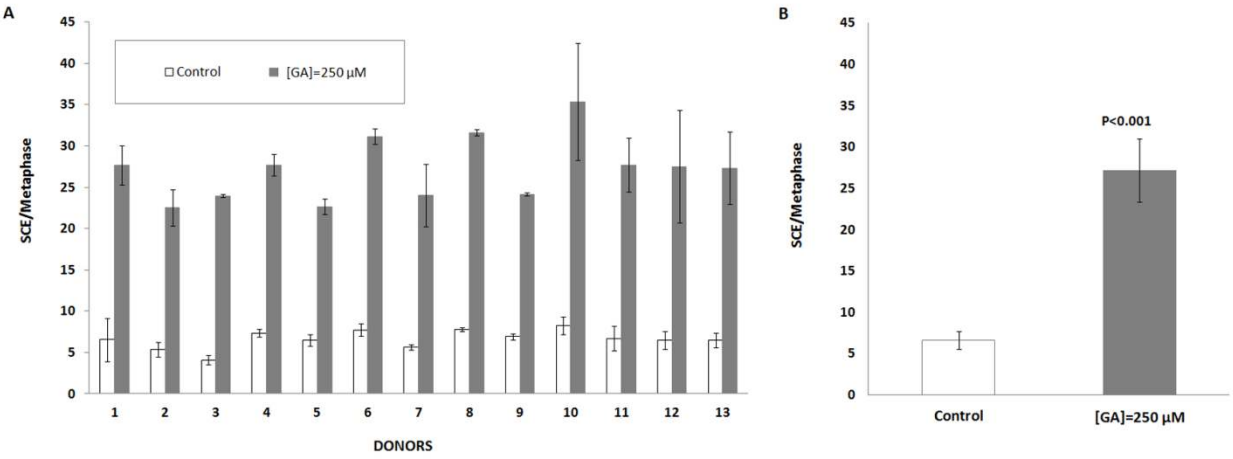



**Fig. 5.3.** Sister chromatid exchanges (SCE/metaphase) induced by glycidamide (GA) in cultured lymphocytes from 13 donors. (**A**) Individual frequencies of SCE/metaphase **(B)** Collective average values of SCE/metaphase. Results are expressed as mean values±SD.

### 5.3.3. DNA-adducts levels induced by AA and GA

The cytogenetic response in terms of DNA adducts (N7-GA-Gua and N3-GA-Ade) was firstly evaluated in stimulated lymphocytes from whole blood culture of two healthy individuals. Different concentrations of AA and GA (up to 2000 µM) were used. The levels of individual GA-DNA adducts obtained are presented in Table V.4. AA adducts were not found at any concentration studied. However, a linear dose-response was observed between the levels of GA exposure and the level of N7-GA-Gua adduct up to GA 750 µM, being this adduct detected at the lower dose studied (10 µM). A high increased in adducts levels were detected for higher doses studied (1000 and 2000 µM) for both donors. GA adducts were not detected in non treated control samples in any of the experiments.

**Table V.4.** Levels of GA-DNA adduct (N7-GA-Gua and N3-GA-Ade) in lymphocytes treated with different concentrations of AA and GA (0-2000 μM) for a 46-hour period for two different donors.

| Test compound | Donor 1 | | Donor 2 | |
|---|---|---|---|---|
| | N7-GA-Gua/ $10^6$ nucleotides | N3-GA-Ade/ $10^6$ nucleotides | N7-GA-Gua/ $10^6$ nucleotides | N3-GA-Ade/ $10^6$ nucleotides |
| **Acrylamide (μM)** | | | | |
| **0** | <LOD | <LOD | <LOD | <LOD |
| **100** | <LOD | <LOD | <LOD | <LOD |
| **250** | <LOD | <LOD | <LOD | <LOD |
| **500** | <LOD | <LOD | <LOD | <LOD |
| **750** | <LOD | <LOD | <LOD | <LOD |
| **1000** | <LOD | <LOD | <LOD | <LOD |
| **2000** | <LOD | <LOD | <LOD | <LOD |
| **Glycidamide (μM)** | | | | |
| **0** | <LOD | <LOD | <LOD | <LOD |
| **10** | 1.8 | <LOD | 1.3 | <LOD |
| **25** | 4.7 | <LOD | 4.3 | <LOD |
| **100** | 18.1 | <LOD | 26.8 | <LOD |
| **250** | 46.6 | <LOD | 55.7 | <LOD |
| **500** | 86.5 | <LOD | 79.3 | <LOD |
| **750** | 98.2 | <LOD | 93.7 | <LOD |
| **1000** | 630.2 | <LOD | 603.6 | 6.20 |
| **2000** | 874.2 | 11.4 | 1051.9 | <LOD |

*Note.* The LOD for N7-GA-Gua and N3-GA-Ade, when assaying 5 μg of DNA, was 0.1 adducts/$10^6$ nucleotides.

N3-GA-Ade, was also analyzed, but only detected for GA treated lymphocytes and for the highest concentrations studied (1000 and 2000 μM for donor 2 and 1, respectively). Further studies must be done in order to clarify these results. For AA treated lymphocytes and for the rest of concentrations, DNA adduct levels were near or below the limit of detection.

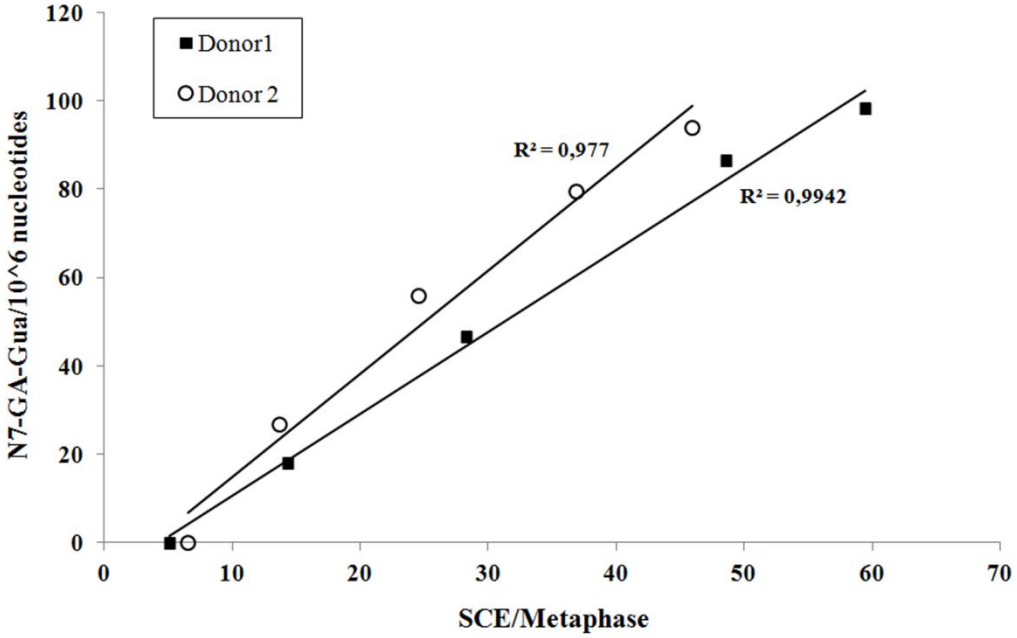



**Fig. 5.4**. Correlation between N7-GA-Gua adduct levels and SCE in lymphocytes exposed to glycidamide (100–750 µM) for two independent donors. Results for SCE/Metaphase are expressed as average values from two independent experiments.

The levels of N7-GA-Gua adducts were compared with the levels of SCEs in AA and GA exposed cultures. A strong linear correlation between the levels of GA exposure and the level of N7-GA-Gua adducts obtained were observed (Figure 5.4) for both donors (r=0.994 and r=0.977).

### 5.3.3. Role of genetic polymorphisms in induction of SCE by GA

The inter-individual variability described above in terms of GA-induced SCE could be related with different polymorphisms in detoxification and DNA- repair genes (Tables V.1 and V.2, respectively). In view of this, the different donors were genotyped for detoxification (*GSTM1*, *GSTT1*, *GSTP1*, *GSTA2* and *EPHX1)* and DNA-repair polymorphisms. For DNA repair, different genes involved in BER (*APEX1*, *MUTYH,*

*OGG1*, *PARP1*, *PARP4*, and *XRCC1*), NER (*ERCC1*, *ERCC2*, *ERCC4*, *ERCC5*, *ERCC6*, *RAD23B* and *XPC*), HRR (*NBS*, *RAD51*, *XRCC2* and *XRCC3*) and NHEJ (*Ku80, Lig4* and *XRCC4*) pathways were also analyzed in order to find out possible associations with GA-induced SCE.

**Table V.5.** Sister-chromatid exchanges induced *in vitro* by glycidamide, considering different genotypes in detoxification genes

| Detoxification pathway | Genotypes | N | SCE/metaphase ± SD | P value |
|---|---|---|---|---|
| **GST** | **GSTM1** | | | |
| | Null | 6 | 21.1 ± 3.9 | |
| | Positive | 7 | 20.1 ± 2.3 | N.S.[(b)] |
| | **GSTT1** | | | |
| | Null | 2 | 18.5 ± 3.3 | |
| | Positive | 11 | 20.9 ± 3.0 | N.S.[(b)] |
| | **GSTP1 (Ile105Val)** | | | |
| | Ile/Ile | 4 | **23.1 ± 3.0** | |
| | Ile/Val | 6 | 20.1 ± 2.2 | N.S.[(a)] |
| | Val/Val | 3 | 18.0 ± 2.4 | N.S.[(a)] |
| | Ile/Val+Val/Val | 9 | **19.4 ± 2.4** | **0.050**[(b)] |
| | **GSTA2 (Pro110Ser)** | | | |
| | Pro/Pro | 13 | 20.6 ± 3.0 | |
| | Pro/Ser | 0 | ----- | |
| | Ser/Ser | 0 | ----- | |
| | **GSTA2 (Ser112Thr)** | | | |
| | Ser/Ser | 4 | 20.0 ± 1.9 | |
| | Ser/Thr | 6 | 22.1 ± 3.5 | N.S.[(a)] |
| | Thr/Thr | 3 | 18.3 ± 2.1 | N.S.[(a)] |
| | Ser/Thr+Thr/Thr | 9 | 20.8 ± 3.5 | N.S.[(b)] |
| | **GSTA2 (Glu210Ala)** | | | |
| | Glu/Glu | 11 | **21.3 ± 2.7** | |
| | Glu/Ala | 2 | **16.7 ± 0.7** | **0.026**[(b)] |
| | Ala/Ala | 0 | --- | |
| **EPH** | **EPHX1 (Tyr113His + His139Arg)** | | | |
| | Low activity | 7 | 20.8 ± 3.7 | |
| | Medium activity | 2 | 19.0 ± 2.6 | N.S.[(a)] |
| | High activity | 3 | 21.8 ± 1.8 | N.S.[(a)] |

N.S. non significant p-value; (a) Kruskal-Wallis Test; (b) Mann-Whitney Test

**Table V.6.** Sister-chromatid exchanges induced *in vitro* by glycidamide, considering different genotypes in DNA repair genes

| REPAIR PATHWAY | Genes | N | SCE/metaphase ± SD | P value |
|---|---|---|---|---|
| **BER** | **XRCC1 (Arg194Trp)** | | | |
| | Arg/Arg | 13 | 20.6 ± 3.0 | |
| | Arg/Trp | 0 | ----- | |
| | Trp/Trp | 0 | ----- | |
| | **XRCC1 (Gln399Arg)** | | | |
| | Arg/Arg | 5 | 20.1 ± 2.9 | |
| | Arg/Gln | 8 | 20.9 ± 3.2 | N.S.[(b)] |
| | Gln/Gln | 0 | ----- | |
| | **OGG1 (Ser326Cys)** | | | |
| | Ser/Ser | 5 | 20.8 ± 1.8 | |
| | Ser/Cys | 8 | 20.4 ± 3.7 | N.S.[(b)] |
| | Cys/Cys | 0 | ----- | |
| | **PARP1 (Val762Ala)** | | | |
| | Val/Val | 7 | 20.6 ± 2.7 | |
| | Val/Ala | 6 | 20.6 ± 3.6 | N.S.[(b)] |
| | Ala/Ala | 0 | ----- | |
| | **PARP4 (Pro1328Thr)** | | | |
| | Pro/Pro | 9 | 20.8 ± 3.5 | |
| | Pro/Thr | 3 | 19.7 ± 2.1 | N.S.[(a)] |
| | Thr/Thr | 1 | 21.0 | N.S.[(a)] |
| | Pro/Thr+Thr/Thr | 4 | 20.0 ±1.9 | N.S.[(b)] |
| | **APEX1 (Asp148Glu)** | | | |
| | Glu/Glu | 3 | 19.2 ± 2.6 | |
| | Glu/Asp | 7 | 19.9 ± 2.4 | N.S.[(a)] |
| | Asp/Asp | 3 | 23.4 ± 3.6 | N.S.[(a)] |
| | Glu/Asp+Asp/Asp | 10 | 21.0 ± 3.1 | N.S.[(b)] |
| | **MUTYH (Gln335His)** | | | |
| | Gln/Gln | 8 | 20.6 ± 3.6 | |
| | Gln/His | 3 | 19.3 ± 1.9 | N.S.[(a)] |
| | His/His | 2 | 22.2 ± 1.7 | N.S.[(a)] |
| | Gln/His+His/His | 5 | 20.4 ± 2.2 | N.S.[(b)] |

**Table V.6.** Continued

| REPAIR PATHWAY | Genes | N | SCE/metaphase ± SD | P value |
|---|---|---|---|---|
| **NER** | **ERCC2 (Lys751Gln)** | | | |
| | Lys/Lys | 3 | 18.9 ± 3.9 | |
| | Lys/Gln | 7 | 20.2 ± 2.1 | N.S.[(a)] |
| | Gln/Gln | 3 | 23.1 ± 3.5 | N.S.[(a)] |
| | Lys/Gln+ Gln/Gln | 10 | 21.1 ± 2.8 | N.S.[(b)] |
| | **ERCC1 (Gln504Lys)** | | | |
| | Gln/Gln | 8 | 20.3 ± 2.8 | |
| | Gln/Lys | 4 | 19.5 ± 1.6 | N.S.[(a)] |
| | Lys/Lys | 1 | 27.1 | N.S.[(a)] |
| | Gln/Lys + Lys/Lys | 5 | 21.0 ± 3.7 | N.S.[(b)] |
| | **ERCC4 (Arg415Gln)** | | | |
| | Arg/Arg | 9 | 20.6 ± 3.2 | |
| | Arg/Gln | 3 | 21.8 ± 1.8 | N.S.[(a)] |
| | Gln/Gln | 1 | 16.2 | N.S.[(a)] |
| | Arg/Gln + Gln/Gln | 4 | 20.4 ± 3.2 | N.S.[(b)] |
| | **ERCC5 (His1104Asp)** | | | |
| | His/His | 8 | 19.9 ± 2.5 | |
| | His/Asp | 5 | 21.6 ± 3.8 | N.S.[(b)] |
| | Asp/Asp | 0 | ----- | |
| | His/Asp + Asp/Asp | | ----- | |
| | **ERCC5 (Cys529Ser)** | | | |
| | Cys/Cys | 12 | 20.6 ± 3.2 | |
| | Cys/Ser | 1 | 20.4 | N.S.[(b)] |
| | Ser/Ser | 0 | ----- | |
| | **ERCC6 (Arg1230Pro)** | | | |
| | Pro/Pro | 11 | 20.3 ± 3.2 | |
| | Pro/Arg | 2 | 22.2 ± 1.7 | N.S.[(b)] |
| | Arg/Arg | 0 | ----- | |
| | **ERCC6 (Gln1413Arg)** | | | |
| | Arg/Arg | 8 | 21.2 ± 3.6 | |
| | Arg/Gln | 5 | 19.5 ± 1.7 | N.S.[(b)] |
| | Gln/Gln | 0 | ----- | |
| | **RAD23B (Ala249Val)** | | | |
| | Ala/Ala | 9 | 19.8 ± 2.8 | |
| | Ala/Val | 4 | 22.4 ± 3.2 | N.S.[(b)] |
| | Val/Val | 0 | ----- | |
| | **XPC (Ala499Val)** | | | |
| | Ala/Ala | 0 | ----- | |
| | Ala/Val | 6 | 21.9 ± 3.3 | |
| | Val/Val | 7 | 19.4 ± 2.4 | N.S.[(b)] |
| | **XPC (Lys939Gln)** | | ----- | |
| | Lys/Lys | 7 | 20.2± 2.4 | |
| | Lys/Gln | 6 | 21.0 ± 3.8 | N.S.[(b)] |
| | Gln/Gln | 0 | ----- | |

**Table V.6.** Continued

| REPAIR PATHWAY | Genes | N | SCE/metaphase ± SD | P value |
|---|---|---|---|---|
| **HRR** | **RAD51 (5'UTR)** | | | |
| | G/G | 5 | 19.5 ± 2.9 | |
| | G/T | 8 | 21.2 ± 3.1 | N.S.[(b)] |
| | T/T | 0 | ----- | |
| | **NBS (Glu185Gln)** | | | |
| | Glu/Glu | 7 | 21.5 ± 3.2 | |
| | Glu/Gln | 4 | 19.5 ± 3.4 | N.S.[(a)] |
| | Gln/Gln | 2 | 19.6 ± 1.7 | N.S.[(a)] |
| | Glu/Gln + Gln/Gln | 6 | 19.5 ± 2.7 | N.S.[(b)] |
| | **XRCC3 (Thr241Met)** | | | |
| | Thr/Thr | 3 | 22.2 ± 5.6 | |
| | Thr/Met | 7 | 20.0 ± 2.3 | N.S.[(a)] |
| | Met/Met | 3 | 20.2 ± 1.6 | N.S.[(a)] |
| | Thr/Met + Met/Met | 10 | 20.1 ±2.0 | N.S.[(b)] |
| | **XRCC2 (Arg188His)** | | | |
| | Arg/Arg | 11 | 20.5 ± 3.1 | |
| | Arg/His | 2 | 20.9 ± 3.6 | N.S.[(b)] |
| | His/His | 0 | ------ | |
| **NHEJ** | **XRCC4 It7G>A** | | | |
| | G/G | 8 | 21.1 ± 3.1 | |
| | G/A | 5 | 19.7± 3.1 | N.S.[(b)] |
| | A/A | 0 | | |
| | **XRCC4 Thr134Ile** | | | |
| | Ile/Ile | 13 | 20.6 ± 3.0 | |
| | Ile/Thr | 0 | ----- | |
| | Thr/Thr | 0 | ----- | |
| | **Lig4Thr9Ile** | | | |
| | Thr/Thr | 10 | 20.9 ± 3.1 | |
| | Thr/Ile | 3 | 19.3 ± 2.7 | N.S.[(b)] |
| | Ile/Ile | 0 | ----- | |
| | **Ku80Ex21-238G>A** | | | |
| | G/G | 5 | 21.3 ± 1.5 | |
| | G/A | 4 | 21.0 ± 5.2 | N.S.[(a)] |
| | A/A | 4 | 19.3 ± 1.8 | N.S.[(a)] |
| | G/A+A/A | 8 | 20.1 ± 3.7 | N.S.[(b)] |
| | **Ku80Ex21+338T>C** | | | |
| | T/T | 10 | 20.8 ± 3.3 | |
| | T/C | 3 | 19.7 ± 2.2 | N.S.[(b)] |
| | C/C | 0 | ----- | |
| | **Ku80Ex21-352C>A** | | | |
| | C/C | 10 | 20.8 ± 3.3 | |
| | C/A | 3 | 19.7 ± 2.2 | N.S.[(b)] |
| | A/A | 0 | ----- | |
| | **Ku80Ex21+466A>G** | | | |
| | A/A | 10 | 20.7 ± 3.0 | |
| | A/G | 3 | 20.2 ± 3.7 | N.S.[(b)] |
| | G/G | 0 | ----- | |

N.S. non significant p-value; (a) Kruskal-Wallis Test; (b) Mann-Whitney Test

The genotypic distribution of the polymorphisms involved in detoxification pathways and its association with the levels of GA-induced SCE is presented in Table V.5. In this table, as in Table V.6, the values of GA-induced SCE correspond to the SCE observed in GA-treated cultures (250 μM) after subtracting the respective background of SCE in non-treated controls. *GSTM1* and *GSTT1* deletion polymorphisms did not influence the level of SCEs induced by GA. Conversely, for *GSTP1* Ile105Val, lymphocytes from wild-type individuals have a higher level of GA-induced SCE than those with at least one variant allele (p=0.050, Table V.5). With respect to *GSTA2,* three SNPs (Pro110Ser, Ser112Thr, Glu210Ala), all non-synonymous were analyzed. For *GSTA2* Glu210Ala polymorphism, no homozygous donor for the variant allele was present in our study group. For this SNP, the level of SCE was lower for lymphocytes of heterozygous individuals (Glu/Ala) when compared with wild-type homozygous individuals (Glu/Glu) (p=0.026). For the Ser112Thr SNP no significant associations were found with SCE data (Table V.5). With regards to the third SNP, *i.e.,GSTA2* Pro110Ser polymorphism, the analysis of a possible association was precluded since all donors had the wild type genotype. *EPHX1* genotypes (Tyr113His and His139Arg) were also analyzed and individuals were classified according to the expected activity of the correspondent enzyme [22, 24]. These SNPs did not show any influence on the level of SCEs induced by GA.

For DNA repair**,** the distribution of genotypic frequencies related with repair pathways (BER, NER, HRR and NHEJ) and its association with SCE levels is presented in Table V.6. For the DNA-repair pathways studied, *i.e.* BER (6 genes), NER (7 genes), HRR (4 genes) and NHEJ (3 genes), no associations with the level of GA-induced SCEs were found.

## 5.4. Discussion

The development of predictive biomarkers for cancer-risk assessment is a challenging issue in food toxicology. The SCE test is well-known for its sensitivity to detect chemical genotoxicants [28]. The usefulness of SCE to evaluate genotoxicity of AA and GA has been previously shown in mammalian cells [29-31]. However, *in vitro* studies using human cells are still lacking. These studies are of utmost importance to predict the potential importance of SCE formation as a valuable cytogenetic toxicological biomarker to be used in the assessment of human risk from exposure to AA. Therefore, in the present work, the levels of SCEs induced by AA and GA were evaluated in human lymphocytes.

PHA-stimulated lymphocytes from whole blood cultures were used throughout this report. This "whole blood" approach has been followed by other authors [32-34] and may be considered more closely related to the physiological situation than isolated lymphocyte cultures, since it encompasses the influence of other factors, such as red blood cells [35], platelets and plasma.

In this study, a slight induction of SCE by AA was found, which was specially associated with the very high and cytotoxic concentration of 2000 μM. In contrast, GA markedly increased the frequency of SCE in a concentration-dependent manner. This is in agreement with our previous study [31], which reported a much higher genotoxic potential of GA when compared with AA, using the same end-point in V79 cells. In addition, the same trend was observed in a recent study from our group, carried out in human whole blood leukocytes analyzed with the comet assay [36].

Additionally, data concerning the levels of DNA-adducts after exposure to AA showed that no DNA-adducts were detected at any concentration studied. In spite of AA-induced DNA adducts not have been detected in the present work, it is important to note that AA highly reacts with protein, specifically haemoglobin, originating haemoglobin adducts [2, 37]. The concentration of haemoglobin adducts reflects the internal doses of AA and GA and this type of adducts are biomarkers for the biological effect [38]. Moreover, several authors have reported positive results of haemoglobin adducts of AA and GA as biomarker of AA exposure [39-42]. On the other hand, our results showed that the major adduct analyzed in human lymphocytes was N7-GA-Gua,

formed by reaction of the DNA with the epoxide metabolite glycidamide. N7-GA-Gua was detected for concentrations as low as 10 μM GA.

Furthermore, N3-GA-Ade was also analyzed but data showed that was formed in much lower amounts and only for the highest concentration studied (1000 and 2000 μM). Similar results were found for DNA adducts measured in liver, brain and testes of rats administered with single oral doses of acrylamide [43, 44].

The cytogenetic end-point evaluated by sister chromatid exchange in stimulated lymphocytes exposed to GA, was compare in same conditions with N7-GA-Gua DNA-adducts obtained. The results showed a strong correlation between these two end-points (Figure 5.4). This correlation is consistent with a mechanism involving the formation of GA-DNA adducts on the induction of SCEs in human lymphocytes after exposure to GA [12] and already suggested by our group in a previous study [31].

In view of this clear genotoxicity of GA, in contrast to AA, the further step of this study was to evaluate the inter-individual response to a GA concentration of 250 μM and to associate it with polymorphisms in detoxification and DNA-repair genes. Although this concentration is much highter than human dietary exposure levels, it was clearly genotoxic for human lymphocytes, without a marked cytotoxicity. Therefore, it enabled us to distinguish differences among individuals. This approach is crucial not only for a thorough understanding of the usefulness of SCE frequency as a cytogenetic biomarker in risk assessment, but also for the identification of genotypes that potentially modulate the genotoxic damage in an AA-exposure scenario.

The induction of SCE by the metabolite GA revealed inter-individual variability (Fig. 5.2.A). The heterogeneity in terms of DNA damage, among individuals with different genotypes, has been described for other xenobiotics and the association with detoxification genes has been reported [32, 45]. The metabolic fate of GA has been extensively reviewed [5, 6]. GA is detoxified by conjugation with glutathione *via* GSTs, or can be hydrolyzed by EPHX [11, 46]. Therefore, the possible association between inter-individual variations in GA-induced DNA damage and polymorphisms in the genes *GSTM1*, *GSTT1*, *GSTP1*, *GSTA2* and *EPHX1* was further analyzed. The SCEs results from this study suggest that *GSTP1* (Ile105Val) and *GSTA2* (Glu210Ala) polymorphisms may influence the detoxification capacity of GA.

The GSTP1 enzyme is the major GST enzyme in blood and metabolizes several diol-epoxides of polycyclic aromatic hydrocarbons (PAH) [10]. The GSTP1 isoenzyme with valine in position 105 has a higher efficiency for conjugation of PAH diol-epoxides [47]. Our results indicate a lower level of cytogenetic damage in lymphocytes from individuals presenting at least one variant allele for *GSTP1* suggesting that a higher conjugation efficiency of GA with GSH could be achieved for the variant forms of *GSTP1*. A similar study by Teixeira *et al* [21] reported that SCE frequencies presented by plastic workers with low exposure to styrene were also related with the *GSTP1* (Ile105Val) polymorphism. Styrene is a small molecule that undergoes metabolization to styrene-7,8-oxide, a low molecular weight epoxide comparable in size to GA. Moreover, the detoxification of toluene di-isocyanate seems to be affected by *GSTP1* 105Val variant allele [48].

With regards to *GSTA2*, there is lack of data on the role of genetic polymorphisms associated with exposure to environmental genotoxicants. *GSTA2* is predominantly expressed in the liver, the major site for detoxification of drugs and xenobiotics [23, 49]. In this study, the heterozygous genotype of *GSTA2* (Glu210Ala) SNP showed a decrease in SCE levels *versus* wild-type individuals (Glu/Glu). This result suggests the possible involvement of *GSTA2* in detoxification of GA. The alpha-class GSTs has commonly been described as one of the most versatile GST classes, since it is responsible for detoxification of a heterogeneous group of compounds [23]. In this context, Ketterer *et al*, pointed out a role for *GSTA2* genotypes in the detoxification of small molecules like cumene hydroperoxide [49, 50]. Previous studies have reported that for the polymorphisms Ser112Thr and Glu210Ala the respective amino acid residues are not at the active site of GSTA2. However, while in the case of Ser112Thr polymorphism the amino acid change did not show an important effect in protein structure, for the Glu210Ala a slight effect in protein function may be observed [49], which may explain the differential results obtained in our study where no differences were found for the Ser112Thr polymorphism. In respect to Pro110Ser polymorphism, which may have some impact on rigidity of the active site [49], no individuals displayed the variant genotype, precluding any analysis.

GSTT1 and GSTM1 enzymes are involved in the metabolism of epoxides and are mainly expressed in liver and blood [10]. Our results show that deletions in the genes *GSTT1* and *GSTM1* do not seem to affect the levels of SCEs. This is in

accordance with previous reports [10, 51] that addressed the involvement of *GSTT1* and *GSTM1* in the toxicokinetics of AA in terms of haemoglobin (Hb)-adducts, concluding that these enzymes do not seem to play a major role in the AA and GA conjugation with glutathione. In contrast, a recent study from Duale *et al* [52] suggested a possible involvement of both *GSTM1* and *GSTT1*. These conflicting results clearly anticipate the need for further studies focusing on GSTs with the use of additional toxicological biomarkers.

The same is valid for the genetic polymorphisms of *EPHX1* for which some association has been recently reported in terms of urinary acrylamide metabolites from workers exposed to acrylamide [53]. EPHX1 catalyzes the hydrolysis of reactive epoxides to their corresponding dihydrodriols, playing an important role in their detoxification. EPHX1 is probably involved in the metabolism of GA to a less reactive and more soluble glyceramide. EPHX1 is expressed in all tissues, including white blood cells [10, 52, 54]. The data reported here did not show any effect of EPHX1 detoxification in relation to GA-induced SCE. Our results are in accordance with Paulson *et al* [10] who previously showed that the chemical inhibition of EPHX1 had no significant effect in the Hb-adducts levels after exposure to AA and GA.

DNA-repair polymorphisms that modulate DNA repair capacity may influence the individual susceptibility to DNA-damaging agents and, therefore, modify cancer risk. In fact, polymorphic variants in DNA-repair genes have been associated with susceptibility for several types of DNA lesions and cancer risk [55]. However, the overall data presented here did not show relevant associations between SCE levels and individual genetic polymorphisms in DNA-repair genes.

In summary, our results show that AA only slightly induced SCEs at a very high concentration. Conversely, GA is clearly genotoxic to cultured human lymphocytes, highlighting the importance of this metabolite. It was also demonstrated that SCE frequency constitutes a sensitive and reliable endpoint to evaluate DNA damage, being a valuable cytogenetic biomarker that could be used, along with other cytogenetic biomarkers (e.g. micronuclei, chromosomal aberrations), to evaluate genotoxic effects in an AA-exposure scenario.

Although these results are based on a small number of blood donors, this exploratory study points out to a possible role for *GSTP1* Ile105Val and *GSTA2*

Glu210Ala on the modulation of the genotoxicity induced by GA. These data provide a further step towards the development of potential susceptibility biomarkers for AA and GA. However, for more conclusive results, studies involving a larger number of individuals should be performed and other polymorphisms and combinations of polymorphisms regarding genes involved in GA detoxification and DNA repair should be further evaluated.

## 5.5. References


1. IARC: **Acrylamide**. In: *Acrylamide In IARC monographs on the evaluation of carcinogenic risk to humans Volume 60*, edn.: International Agency for Research on Cancer, Lyon; 1994: 389-433.

2. Carere A: **Genotoxicity and carcinogenicity of acrylamide: a critical review**. *AnnIstSuperSanita* 2006, **42**(2):144-155.

3. Parzefall W: **Minireview on the toxicity of dietary acrylamide**. *Food ChemToxicol* 2008, **46**(4):1360-1364.

4. Stadler RH, Blank I, Varga N, Robert F, Hau J, Guy PA, Robert MC, Riediker S: **Acrylamide from Maillard reaction products**. *Nature* 2002, **419**(6906):449-450.

5. Friedman M: **Chemistry, biochemistry, and safety of acrylamide. A review**. *JAgricFood Chem* 2003, **51**(16):4504-4526.

6. Rice JM: **The carcinogenicity of acrylamide**. *MutatRes* 2005, **580**(1-2):3-20.

7. Zeiger E, Recio L, Fennell TR, Haseman JK, Snyder RW, Friedman M: **Investigation of the low-dose response in the in vivo induction of micronuclei and adducts by acrylamide**. *ToxicolSci* 2009, **107**(1):247-257.

8. Tareke E, Rydberg P, Karlsson P, Eriksson S, Tornqvist M: **Analysis of acrylamide, a carcinogen formed in heated foodstuffs**. *Journal of agricultural and food chemistry* 2002, **50**(17):4998-5006.

9. Dybing E, Sanner T: **Risk assessment of acrylamide in foods**. *ToxicolSci* 2003, **75**(1):7-15.

10. Paulsson B, Rannug A, Henderson AP, Golding BT, Tornqvist M, Warholm M: **In vitro studies of the influence of glutathione transferases and epoxide hydrolase on the detoxification of acrylamide and glycidamide in blood**. *MutatRes* 2005, **580**(1-2):53-59.

11. Gargas ML, Kirman CR, Sweeney LM, Tardiff RG: **Acrylamide: Consideration of species differences and nonlinear processes in estimating risk and safety for human ingestion**. *Food ChemToxicol* 2009, **47**(4):760-768.

12. Gamboa da Costa G, Churchwell MI, Hamilton LP, Von Tungeln LS, Beland FA, Marques MM, Doerge DR: **DNA adduct formation from acrylamide via conversion to glycidamide in adult and neonatal mice**. *Chemical research in toxicology* 2003, **16**(10):1328-1337.

13. Wilson KM, Mucci LA, Rosner BA, Willett WC: **A prospective study on dietary acrylamide intake and the risk for breast, endometrial, and ovarian cancers**. *Cancer EpidemiolBiomarkers Prev* 2010, **19**(10):2503-2515.

14. Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA: **A prospective study of dietary acrylamide intake and the risk of endometrial, ovarian, and breast cancer**. *Cancer EpidemiolBiomarkers Prev* 2007, **16**(11):2304-2313.

15. Olesen PT, Olsen A, Frandsen H, Frederiksen K, Overvad K, Tjonneland A: **Acrylamide exposure and incidence of breast cancer among postmenopausal women in the Danish Diet, Cancer and Health Study**. *IntJCancer* 2008, **122**(9):2094-2100.

16. Perry P, Wolff S: **New Giemsa method for the differential staining of sister chromatids**. *Nature* 1974, **251**(5471):156-158.

17. Dean BJ, Danford N: **Assays for the detection of chemically-induced chromosome damage in cultured mammalian cells.** In: *Mutagenicity Testing A pratical approach* edn. Edited by Venitt S, Parry JM. Oxford: IRL Press; 1984: 187-232.

18. Lin DX, Tang YM, Peng Q, Lu SX, Ambrosone CB, Kadlubar FF: **Susceptibility to esophageal cancer and genetic polymorphisms in glutathione S-transferases T1, P1, and M1 and cytochrome P450 2E1**. *Cancer EpidemiolBiomarkers Prev* 1998, **7**(11):1013-1018.

19. Costa S, Coelho P, Costa C, Silva S, Mayan O, Santos LS, Gaspar J, Teixeira JP: **Genotoxic damage in pathology anatomy laboratory workers exposed to formaldehyde**. *Toxicology* 2008, **252**(1-3):40-48.

20. Gaspar J, Rodrigues S, Gil OM, Manita I, Ferreira TC, Limbert E, Goncalves L, Pina JE, Rueff J: **Combined effects of glutathione S-transferase polymorphisms and thyroid cancer risk**. *Cancer GenetCytogenet* 2004, **151**(1):60-67.

21. Teixeira JP, Gaspar J, Silva S, Torres J, Silva SN, Azevedo MC, Neves P, Laffon B, Mendez J, Goncalves C *et al*: **Occupational exposure to styrene: modulation of cytogenetic damage and levels of urinary metabolites of styrene by polymorphisms in genes CYP2E1, EPHX1, GSTM1, GSTT1 and GSTP1**. *Toxicology* 2004, **195**(2-3):231-242.

22. Sarmanova J, Tynkova L, Susova S, Gut I, Soucek P: **Genetic polymorphisms of biotransformation enzymes: allele frequencies in the population of the Czech Republic**. *Pharmacogenetics* 2000, **10**(9):781-788.

23. Silva SN, Azevedo AP, Teixeira V, Pina JE, Rueff J, Gaspar JF: **The role of GSTA2 polymorphisms and haplotypes in breast cancer susceptibility: a case-control study in the Portuguese population**. *OncolRep* 2009, **22**(3):593-598.

24. Costa C, Teixeira JP, Silva S, Roma-Torres J, Coelho P, Gaspar J, Alves M, Laffon B, Rueff J, Mayan O: **Cytogenetic and molecular biomonitoring of a Portuguese population exposed to pesticides**. *Mutagenesis* 2006, **21**(5):343-350.

25. Bastos HN, Antao MR, Silva SN, Azevedo AP, Manita I, Teixeira V, Pina JE, Gil OM, Ferreira TC, Limbert E *et al*: **Association of polymorphisms in genes of the homologous recombination DNA repair pathway and thyroid cancer risk**. *Thyroid* 2009, **19**(10):1067-1075.

26. Conde J, Silva SN, Azevedo AP, Teixeira V, Pina JE, Rueff J, Gaspar JF: **Association of common variants in mismatch repair genes and breast cancer susceptibility: a multigene study**. *BMCCancer* 2009, **9**:344.

27. Gomes BC, Silva SN, Azevedo AP, Manita I, Gil OM, Ferreira TC, Limbert E, Rueff J, Gaspar JF: **The role of common variants of non-homologous end-joining repair genes XRCC4, LIG4 and Ku80 in thyroid cancer risk**. *OncolRep* 2010, **24**(4):1079-1085.

28. Banerjee A, Benedict WF: **Production of sister chromatid exchanges by various cancer chemotherapeutic agents**. *Cancer Res* 1979, **39**(3):797-799.

29. Dearfield KL, Douglas GR, Ehling UH, Moore MM, Sega GA, Brusick DJ: **Acrylamide: a review of its genotoxicity and an assessment of heritable genetic risk**. *MutatRes* 1995, **330**(1-2):71-99.

30. Glatt H, Schneider H, Liu Y: **V79-hCYP2E1-hSULT1A1, a cell line for the sensitive detection of genotoxic effects induced by carbohydrate pyrolysis products and other food-borne chemicals**. *MutatRes* 2005, **580**(1-2):41-52.

31. Martins C, Oliveira NG, Pingarilho M, Gamboa da CG, Martins V, Marques MM, Beland FA, Churchwell MI, Doerge DR, Rueff J *et al*: **Cytogenetic damage induced by acrylamide and glycidamide in mammalian cells: correlation with specific glycidamide-DNA adducts**. *ToxicolSci* 2007, **95**(2):383-390.

32. Ramos DL, Gaspar JF, Pingarilho M, Gil OM, Fernandes AS, Rueff J, Oliveira NG: **Genotoxic effects of doxorubicin in cultured human lymphocytes with different glutathione S-transferase genotypes**. *MutatRes* 2011, **724**(1-2):28-34.

33. Thielen S, Baum M, Hoffmann M, Loeppky RN, Eisenbrand G: **Genotoxicity of glycidamide in comparison to (+/-)-anti-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide and alpha-acetoxy-N-nitroso-diethanolamine in human blood and in mammalian V79-cells**. *Molecular nutrition & food research* 2006, **50**(4-5):430-436.

34. Baum M, Fauth E, Fritzen S, Herrmann A, Mertes P, Merz K, Rudolphi M, Zankl H, Eisenbrand G: **Acrylamide and glycidamide: genotoxic effects in V79-cells and human blood**. *Mutation research* 2005, **580**(1-2):61-69.

35. Rueff J, Chiapella C, Chipman JK, Darroudi F, Silva ID, Duverger-van BM, Fonti E, Glatt HR, Isern P, Laires A *et al*: **Development and validation of alternative metabolic systems for mutagenicity testing in short-term assays**. *Mutation Research* 1996, **353**(1-2):151-176.

36. Pingarilho M, Oliveira NG, Martins C, Fernandes AS, Lima JP, Rueff J, Gaspar JF: **Genetic polymorphisms in detoxification and DNA repair genes and susceptibility to glycidamide-induced DNA damage**. *Journal of Toxicology and Environmental Health A* 2012, **75**:920-933.

37. Klaunig JE: **Acrylamide carcinogenicity**. *JAgricFood Chem* 2008, **56**(15):5984-5988.

38. Hartmann EC, Boettcher MI, Schettgen T, Fromme H, Drexler H, Angerer J: **Hemoglobin adducts and mercapturic acid excretion of acrylamide and glycidamide in one study population**. *Journal of agricultural and food chemistry* 2008, **56**(15):6061-6068.

39. Bergmark E: **Hemoglobin adducts of acrylamide and acrylonitrile in laboratory workers, smokers and nonsmokers**. *Chemical research in toxicology* 1997, **10**(1):78-84.

40. Bergmark E, Calleman CJ, He F, Costa LG: **Determination of hemoglobin adducts in humans occupationally exposed to acrylamide**. *Toxicology and applied pharmacology* 1993, **120**(1):45-54.

41. Fennell TR, Snyder RW, Krol WL, Sumner SC: **Comparison of the hemoglobin adducts formed by administration of N-methylolacrylamide and acrylamide to rats**. *Toxicological sciences : an official journal of the Society of Toxicology* 2003, **71**(2):164-175.

42. Schettgen T, Weiss T, Drexler H, Angerer J: **A first approach to estimate the internal exposure to acrylamide in smoking and non-smoking adults from Germany**. *International journal of hygiene and environmental health* 2003, **206**(1):9-14.

43. Maniere I, Godard T, Doerge DR, Churchwell MI, Guffroy M, Laurentie M, Poul JM: **DNA damage and DNA adduct formation in rat tissues following oral administration of acrylamide**. *Mutation research* 2005, **580**(1-2):119-129.

44. Doerge DR, da Costa GG, McDaniel LP, Churchwell MI, Twaddle NC, Beland FA: **DNA adducts derived from administration of acrylamide and glycidamide to mice and rats**. *Mutation research* 2005, **580**(1-2):131-141.

45. Silva MC, Gaspar J, Duarte SI, Faber A, Rueff J: **GSTM1, GSTT1, and GSTP1 genotypes and the genotoxicity of hydroquinone in human lymphocytes**. *EnvironMolMutagen* 2004, **43**(4):258-264.

46. Paulsson B, Warholm M, Rannug A, Tornqvist M: **In vitro studies of the influence of certain enzymes on the detoxification of acrylamide and glycidamide in blood**. *AdvExpMedBiol* 2005, **561**:127-133.

47. Hu X, O'Donnell R, Srivastava SK, Xia H, Zimniak P, Nanduri B, Bleicher RJ, Awasthi S, Awasthi YC, Ji X *et al*: **Active site architecture of polymorphic forms of human glutathione S-transferase P1-1 accounts for their enantioselectivity and disparate activity in the glutathione conjugation of 7beta,8alpha-dihydroxy-9alpha,10alpha-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene**. *BiochemBiophysResCommun* 1997, **235**(2):424-428.

48. Broberg KE, Warholm M, Tinnerberg H, Axmon A, Jonsson BA, Sennbro CJ, Littorin M, Rannug A: **The GSTP1 Ile105 Val polymorphism modifies the metabolism of toluene di-isocyanate**. *Pharmacogenetics and genomics* 2010, **20**(2):104-111.

49. Tetlow N, Board PG: **Functional polymorphism of human glutathione transferase A2**. *Pharmacogenetics* 2004, **14**(2):111-116.

50. Ketterer B: **Glutathione S-transferases and prevention of cellular free radical damage**. *Free RadicRes* 1998, **28**(6):647-658.

51. Doroshyenko O, Fuhr U, Kunz D, Frank D, Kinzig M, Jetter A, Reith Y, Lazar A, Taubert D, Kirchheiner J *et al*: **In vivo role of cytochrome P450 2E1 and glutathione-S-transferase activity for acrylamide toxicokinetics in humans**. *Cancer EpidemiolBiomarkers Prev* 2009, **18**(2):433-443.

52. Duale N, Bjellaas T, Alexander J, Becher G, Haugen M, Paulsen JE, Frandsen H, Olesen PT, Brunborg G: **Biomarkers of human exposure to acrylamide and relation to polymorphisms in metabolizing genes**. *ToxicolSci* 2009, **108**(1):90-99.

53. Huang YF, Chen ML, Liou SH, Chen MF, Uang SN, Wu KY: **Association of CYP2E1, GST and mEH genetic polymorphisms with urinary acrylamide metabolites in workers exposed to acrylamide**. *ToxicolLett* 2011, **203**(2):118-126.

54. Fretland AJ, Omiecinski CJ: **Epoxide hydrolases: biochemistry and molecular biology**. *ChemBiolInteract* 2000, **129**(1-2):41-59.

55. Rueff J, Gaspar J, Kranendonk M: **DNA polymorphisms as modulators of genotoxicity and cancer**. *BiolChem* 2002, **383**(6):923-932.

# Chapter 6

## Genetic polymorphisms in detoxification and DNA repair genes and susceptibility to glycidamide-induced DNA damage.

This chapter was adapted from:

"Genetic polymorphisms in detoxification and DNA repair genes and susceptibility to glycidamide-induced DNA damage." Pingarilho M, Oliveira NG, Martins C, Fernandes AS, Lima JP, Rueff J and Gaspar JF. Journal of Toxicology and Environmental Health, Part A (2012) 75, 920-933.

.

**Abstract**

Acrylamide (AA) is a probable human carcinogen formed in carbohydrate-rich foodstuffs upon heating. Glycidamide (GA), the AA metabolite formed by epoxidation is considered the ultimate genotoxic agent. In this study, the *in vitro* genotoxic potential of AA and GA in human whole blood leukocytes was compared using the alkaline comet assay. Although AA did not induce DNA damage in the concentrations tested (up to 1000 μM), GA markedly increased the %Tail DNA at concentrations ≥ 250 μM ($p<0.005$). Further, this study addressed the role of genetic polymorphisms in key genes involved in metabolism and DNA repair pathways (BER, NER, HRR and NHEJ) on GA-induced genotoxicity assessed by the alkaline comet assay. The results obtained suggested associations between DNA damage and polymorphisms of BER (*MUTYH* Gln335His and *XRCC1* Gln399Arg) and NER (*XPC* Ala499Val and Lys939Gln) genes, either alone or in combination.

## 6.1. Introduction

Acrylamide (AA) is a suspected human carcinogen classified as a probable human carcinogen by IARC [1]. AA is an important industrial monomer mainly used for the production of polyacrylamides since the mid-1950s. These compounds gained importance in many field application, namely in wastewater treatment, as flocculants for clarifying drinking-water, as flow control agents in oil-well operations and in gels used for laboratory techniques [2-6].

Acrylamide is also a component of tobacco smoke (1-2 µg/cigarette) and is used in cosmetic additives, including creams, body lotions and shampoos [2]. Further, the general population is exposed to varying amounts of AA via diet [4, 7]. In fact, AA may be generated from food components during heat treatment as a result of the Maillard reaction between an amino acid, primarily asparagine (the major amino acid in potatoes and cereals) and reducing sugars, such as glucose [8]. The extent of AA formation strongly depends on the heating conditions and type and concentration of certain foodstuffs [9]. AA is found at relatively high concentrations (micrograms to milligrams per kilogram) in common food items such as French fries, potato crisp, crisp bread, bread, coffee and cookies [10-12]. Average intake was estimated to be in the range of 0.3 to 0.8 µg AA/Kg body weight (bw)/d for developed countries, corresponding to aproximately 21-26 µg/day for a 70-Kg person, although in children the oral exposure may be two- to threefold highter [2].

Concerns on the health risks of AA for the general population have been raised, since AA is a known rodent neurotoxicant and multisite carcinogen [13, 14]. In fact, chronic studies in rodent models demonstrated that AA is carcinogenic at different organ sites, including the mammary gland [6, 7]. AA is metabolized to the epoxide derivative glycidamide (GA), presumably mediated by cytochrome P450 2E1 and postulate to be the ultimate metabolite that plays a critical role in AA-induced genotoxicity [15-17]. While AA possesses high capacity to bind proteins, GA has a high affinity to bind DNA, generating DNA-adducts [2]. In this context, different DNA adducts have been quantified, being N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua), the predominant adduct [18-20].

Epidemiological data in human populations exposed to AA are conflicting with respect to cancer risk assessment [21-25]. Genetic variability in metabolism (detoxification) and DNA repair genes might influence individual susceptibility to cancer [26, 27]. It is therefore important to correlate genetic polymorphisms with DNA damage using adequate biomarkers of genotoxicity.

Primary DNA damage in human cells may be evaluated by the single-cell gel electrophoresis (SCGE) or comet assay, a simply, sensitive, versatile, rapid and economic method [28]. The comet assay, under alkaline conditions, detects single- and double-strand breaks, incomplete repair sites, alkali-labile sites and DNA cross-linking in any eukariotic cell population [29]. The aim of this study was to assess the *in vitro* genotoxicity of AA and GA in human peripheral blood leukocytes (PBL) using the comet assay. Further, this study aimed to identify possible associations between DNA damage and biomarkers of susceptibility concerning individual genetic polymorphisms in key metabolism and repair genes.

## 6.2.Materials and Methods

### 6.2.1.Chemicals

Acrylamide (AA; CAS registry number 79-06-1, ≥99.5% pure) was purchased from Fluka (Buchs, Switzerland). Glycidamide (CAS Registry Number 5694-00-8, >98.5% pure, containing ~1% AA) was obtained from Toronto Research Chemicals (North York, Ontario, Canada). Fetal calf serum (FCS), Ham's F-10 medium, penicillin-streptomycin solution, phosphate-buffered saline pH 7.4 (PBS), $Na_2EDTA$, Trizma base, Triton X-100, low melting point (LMP) agarose and ethidium bromide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), ethanol, sodium chloride and sodium hydroxide were acquired from Merck (Darmstadt, Germany). Agarose multi-purpose, used as normal melting point (NMP) agarose, was obtained from Bioline (London, UK). Heparin was acquired from B. Braun (Lisbon, Portugal).

### 6.2.2. Blood Sample Collection

Peripheral blood samples were obtained from 25 healthy donors (18 female and 7 male, mean ages 27.1 ± 4.5). Samples of 10 ml were collected under sterile conditions by venipuncture in heparinized tubes. All samples were coded and analyzed under blind conditions. All donors were informed about the aim and experimental details of the study and an informed consent was obtained from all participating subjects prior to the start of the study. Each participant completed one standardized questionnaire about health history, lifestyle, alcohol consumption, medication usage, family history of cancer, exposure to indoor/outdoor pollutants and dietary habits. Donors were all nonsmokers. Ethical approval for this study was obtained from the institutional Ethical Board of the Faculty of Medical Sciences of New University of Lisbon.

### 6.2.3. Comet assay

The comet assay was performed under alkaline conditions essentially according to the procedures of Singh *et al*.[30] and Busto *et al*, [31] with minor modifications. Nonstimulated whole blood (125 µl) was suspended in 1125 µl of Ham's F-10 medium supplemented with 24 % (v/v) of FCS, 1% (v/v) antibiotics (final concentrations of 100 UI/mL penicillin G and 100 µg/mL streptomycin), 1% (v/v) L-glutamine and 1% (v/v) heparin in a 10 ml sterile tube. Blood cultures were then exposed to different concentrations of AA or GA, ranging from 10 to 1000 µM and incubated for 1 hour at 37 ºC. Afterwards, cells were washed twice with fresh culture medium and then collected by centrifugation (1200 x *g* for 5 min). Seven microliters of cell suspension were ressuspended in 70 µl of 0.5 % LMP agarose in PBS (pH=7.4) and dropped onto a frosted slide precoated with a layer of 1 % NMP agarose. Slides were placed at 4 ºC for 20 min and allowed to solidify. Cover slips were then removed and slides immersed in a 4 ºC freshly prepared lysing solution (2.5 M NaCl, 100 mM $Na_2EDTA$, 10 mM Trizma base, 1% Triton X-100, 10 % DMSO, pH=10) overnight in the dark. After lysis, slides were washed twice with ice-cold bidistilled water for 10 min and then slides were randomly placed on a horizontal electrophoresis tank at 4 ºC. The tank was filled with freshly made alkaline electrophoresis solution (1mM EDTA, 300 mM NaOH, pH≥13) to cover the slides. The slides were left in electrophoresis solution for 20 min in the dark

to allow DNA unwinding and alkali-labile sites expression. Electrophoresis was carried out for 20 min at 25 V (1.0 V/cm) and 300 mA. The slides were washed gently thrice at 5-min intervals with a neutralizing buffer (400 mM Tris, pH=7.5) to remove excess alkali and reagents. After neutralization, slides were passed through 50, 70 and 100 % solutions of ethanol for 5 min each, drained, stained with 100 µl of ethidium bromide solution (20 µg/ml), and covered with cover slips. Slides were stored at 4 ºC in humidified containers until analysis. Two independent experiments were conducted in duplicate for the dose-response curve (four slides per data point) and two replicate blood cultures were carried out for each donor.

The comets were observed at a 400 x magnification with a fluorescent microscope (Leica DMLB 100S) equipped with an excitation filter of 530-545 nm and a suppressor filter of 610-675 nm. Fifty individual cells from each slide were randomly analyzed, giving a total of 200 cells analyzed per data point. The percentage of DNA in Tail (% Tail DNA) of comets was measured to assess the extent of DNA damage.

Image analysis was performed using the *Tri Tek Comet Score*$^{TM}$ *v 1.5*, using a 65 *% cutoff*. The median of the % DNA in Tail was used as representative value for each subject, and the media of medians was used for statistical analysis [32].

### 6.2.4. DNA extraction

Genomic DNA was obtained from 250 µl of whole blood using a commercially available kit, according to the manufacturer instructions (QIAamp DNA extraction kit; Qiagen, Hilden, Germany). Each DNA sample was stored at –20 °C until analysis.

### 6.2.5. Genotyping

This study included polymorphisms in genes associated with metabolism, specifically glutathione S-transferases (*GSTM1, GSTT1, GSTP1, GSTA2*) and *EPHX1*. In addition, polymorphisms in DNA repair genes from the base excision repair (BER), nucleotide excision repair (NER), homologous recombination repair (HRR) and non-homologous end-joining repair (NHEJ) pathways were studied.

#### 6.2.5.1. Detoxification pathways

*GSTM1* and *GSTT1* genotyping for gene deletions were carried out by a multiplex PCR as described by Lin *et al.* [33] with minor modifications described in Costa *et al* [26] and as described in Chapter 5 (5.2.9.1).

The genotyping of *GSTP1* Ile105Val (rs1695), *EPHX1* Tyr113His (rs1051740) and His139Arg (rs2234922), *GSTA2* Glu210Ala (rs6577), were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP), as described in Chapter 5 (5.2.9.1).

#### 6.2.5.2. DNA repair pathways

The genotyping of *XRCC2* Arg188His (rs3218536), *XRCC3* Thr241Met (rs861539) and *XPC* Lys939Gln (rs2228001) and Ala499Val (rs2228000) were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP) as described in Chapter 5 (5.2.9.2).

*APEX* Asp148Glu (rs1130409), *ERCC1* Gln504Lys (rs3212986), *ERCC2* Lys751Gln (rs13181), *ERCC4* Arg415Gln (rs1800067), *ERCC5* Cys529Ser (rs2227869) and His1104Asp (rs17655), *ERCC6* Gln1413Arg (rs2228529) and Arg1230Pro (rs4253211), *GSTA2* Pro110Ser (rs2234951) and Ser112Thr (rs2180314), *Ku80* Ex21-238G→A (rs2440); Ex21+338T→C (rs1051677), Ex21-352C→A (rs6941), Ex21+466A→G (rs1051685), *LIG4* Thr9Ile (rs1805388), *MUTYH* Gln335His (rs3219489), *NBS1* Glu185Gln (rs1805794), *OGG1* Ser326Cys (rs1052133), *PARP1* Val762Ala (rs1136410), *PARP4* Gly1280Arg (rs13428) and Pro1328Thr (rs1050112), *RAD23B* Ala249Val (rs1805329), *RAD51* 5'UTR (rs1801321), *XRCC1* Gln399Arg (rs25487) and *XRCC4* It7G>A (rs1805377) and Thr134Ile (rs28360135) polymorphisms were genotyped by Real-Time PCR (AB7300), using TaqMan SNP Genotyping Assays from Applied Biosystems, according to the manufacturer's recommendations and to previous reports from our group [34-37] with minor modifications and as described in Chapter 5 (5.2.9.2). Genotype determinations were carried out twice (all samples for multiplex and PCR-RFLP and 20% of samples for

Real-Time PCR) in independent experiments and all the inconclusive samples were reanalyzed.

### 6.2.6. Statistical analysis

The Kolmogorov-Smirnof test was used to verify the normality of the continuous variables (% Tail DNA). For the variables with a normal distribution the homogeneity of the variances was evaluated using the Levene test and the association of % Tail DNA (value obtained subtracting the tail DNA percent value for background) and the different genotypes obtained was evaluated by Student's *t*-test. The level of significance considered was $p \leq 0.05$. All analyses were performed with the SPSS statistical package (version 17, SPSS Inc., Chicago, IL, USA).

## 6.3. Results:

The concentration-response profile of AA and GA in terms of DNA damage was evaluated by the comet assay in PBL from one healthy individual, and the results are depicted in Figure 6.1. For AA, no marked changes in % Tail DNA were found for the concentrations studied (10-1000 µM). Conversely, GA elevated the % Tail DNA in a concentration-dependent manner for concentrations ranging from 10 to 1000 µM, and this rise was more pronounced and significant for concentrations higher than 250 µM. GA at 1000 µM increased the % Tail DNA approximately 4.4-fold compared to controls. Based on the concentration-response curve of GA, the concentration of 250 µM was selected to be used in the comet assay to all 25 individuals. This concentration provided sufficient sensitivity to discriminate between individual responses, increasing the % Tail DNA in about 2.6 fold in relation to control PBL (Figure 6.1).

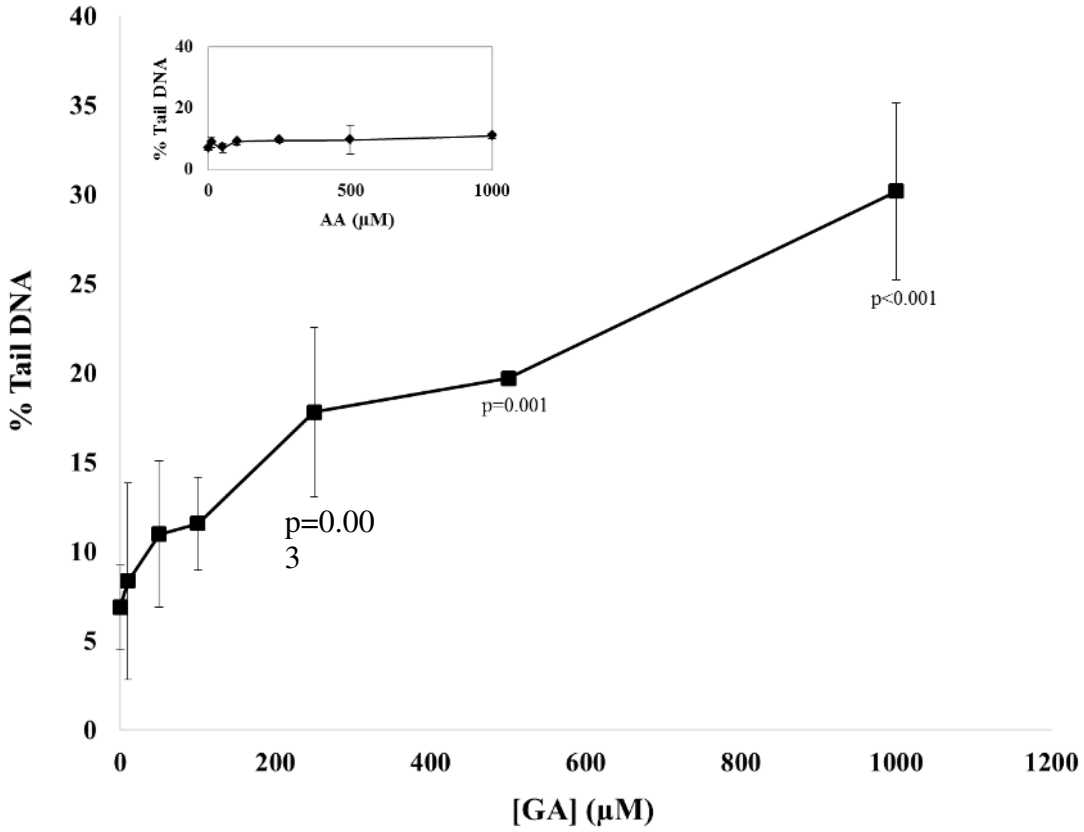



**Figure 6.1.** Percent DNA in Tail (%Tail DNA) induced by AA and GA in whole blood leucocytes from a healthy donor. Results are expressed as the mean ± SD from two independent experiments

The individual effects of 250 µM of GA in terms of % Tail DNA obtained for the 25 individuals are presented in Figure 6.2 (A). The collective average values and respective SD are presented in Figure 6.2 (B). The mean level of % Tail DNA was 11.4 ± 4.9 for untreated PBL of all donors, while it rose 1.7-fold after 1 h of exposure to GA to a mean value of 19.0 ± 6.0 %. From Fig 6.2, it is also clear that the results obtained for individual donors treated with GA showed interindividual variability. In fact, some donors displayed a much more pronounced response (e.g. donors 1 and 23) to GA insult than other donors (e.g. 11 and 12), that only numerically increased the % Tail DNA.

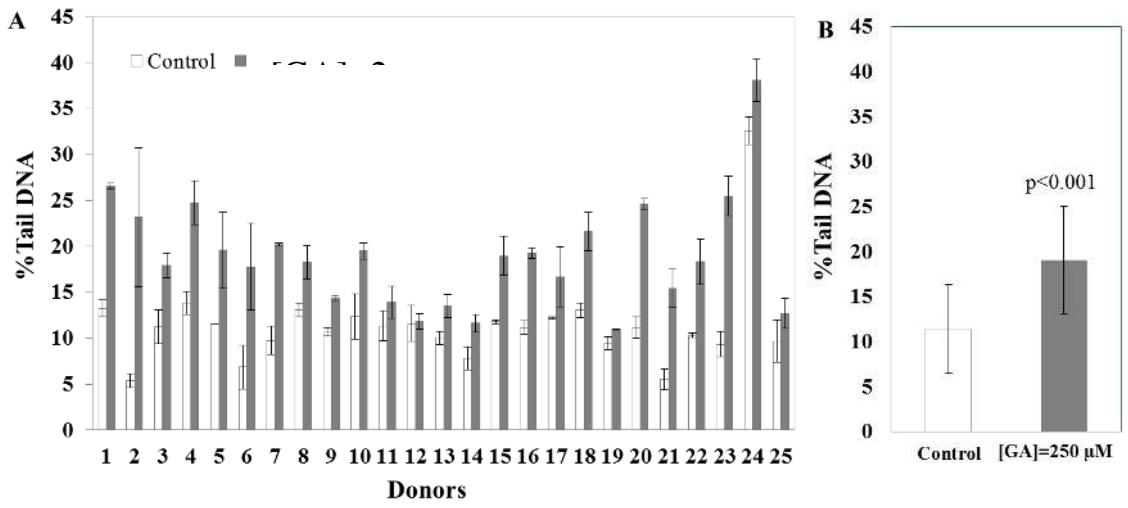



**Figure 6.2.** Percent DNA in Tail (% Tail DNA) induced by GA in whole blood leukocytes for 25 healthy donors. **(A)** Individual values of % Tail DNA. Results are expressed as mean ± SD from two replicates. **(B)** Collective values of % Tail DNA. Results are expressed as mean values ± SD.

This variability in terms of GA-induced % Tail DNA may be correlated with different polymorphisms in detoxification and repair genes. In this context, the study subjects were therefore characterized with respect to genetic polymorphisms in detoxification (*GSTM1*, *GSTT1*, *GSTP1*, *GSTA2* and *EPHX1*), and DNA repair pathways genes. Among these repair pathways, genes from BER (*XRCC1*, *OGG1*, *PARP1*, *PARP4*, *APEX1* and *MUTYH*), NER (*ERCC2*, *ERCC1*, *ERCC4*, *ERCC5*, *ERCC6*, *RAD23B* and *XPC*), HRR (*RAD51*, *NBS*, *XRCC3* and *XRCC2*) and NHEJ (*XRCC4*, *Lig4* and *Ku80*) were evaluated. Afterwards, genotyping data was integrated with the GA-induced % Tail DNA obtained from the comet assay, in order to establish possible associations between those polymorphisms and DNA damage.

The genotypic distribution of polymorphisms involved in metabolism and repair pathways and its association with the levels of GA-induced % Tail DNA are presented in Tables VI.1 to VI.5. In these tables, the values of GA-induced % Tail DNA were calculated by subtracting the % Tail DNA value for the non treated controls (background) from the % Tail DNA value observed in GA treated cultures (250 µM).

In relation to the metabolism polymorphisms studied involved in the detoxification of xenobiotics, no significant correlations between GA-induced %Tail DNA values and genotypic frequencies of polymorphisms were observed (Table VI.1).

The same was noted for the haplotypes generated from *GSTA2* polymorphisms Pro110Ser, Ser112Thr and Glu210Ala (data not shown).

Polymorphisms in DNA repair pathways included 6 genes involved in BER (Table VI.2). However, it is important to note that *PARP4* polymorphisms Pro1328Thr and Gly1280Arg are in linkage disequilibrium. In view of this only *PARP4* Pro1328Thr was studied. It was noted that for *MUTYH* Gln335His there was a marked increase in GA-induced % Tail DNA in homozygous individuals for variant genotype (11.9 ± 1.8%) when compared with heterozygous individuals (6.1 ± 1.7%). However, in relation to other BER SNP studied, no significant associations were found.

In order to further characterize the influence of BER in GA-induced genotoxicity, the combination of different polymorphisms of this pathway was also included in this study. This approach allowed the identification of haplotypes potentially associated with DNA damage induced by GA (Table VI.6). These haplotypes included *XRCC1* Gln399Arg and *MUTYH* Gln335His. A significant rise in GA-induced % Tail DNA was obtained for ArgArg+ArgGln/HisHis (11.9 ± 1.8%) compared with ArgArg+ArgGln/GlnGln+GlnHis (7.0 ± 4.5%) and with GlnGln/GlnGln+GlnHis (4.3±1.2%).

The NER polymorphisms *per se* did not present any significant association with GA-induced DNA damage (Table VI.3). However, haplotypes generated from *XPC* Ala499Val and Lys939Gln (Table VI.7) showed a significant association when comparing AlaVal/LysGln with ValVal/LysGln (2.8 ± 3.7% vs. 9.1± 3.3%, $p<0.05$). Other associations between SNPs of a single gene of this pathway (e.g. *ERCC5* Asp1104His and Cys529Ser polymorphisms) did not reveal any significant association (data not shown).

For the other DNA repair pathways studied, that is HRR (four genes) and NHEJ (three genes) no statistically significant associations were found between the genotyping frequencies and the GA-induced % Tail DNA (Tables VI.4 and VI.5). In addition, no marked associations were found for NHEJ haplotypes and DNA damage attributable to GA (data not shown).

**Table VI.1.** Percent DNA in Tail (%Tail DNA) induced by GA in whole blood leukocytes from 25 healthy individuals considering different genotypes of genes involved in metabolism.

| Detoxification pathway | Genotypes | N | % Tail DNA ± SD |
|---|---|---|---|
| **GST** | **GSTM1** | | |
| | Positive | 13 | 8.9 ± 4.6 |
| | Null | 12 | 6.3 ± 4.0 |
| | **GSTT1** | | |
| | Positive | 22 | 7.3 ± 4.6 |
| | Null | 3 | 9.8 ± 3.1 |
| | **GSTP1 (Ile105Val)** | | |
| | Ile/Ile | 12 | 8.1 ± 5.5 |
| | Ile/Val | 11 | 7.0 ± 3.7 |
| | Val/Val | 2 | 8.0 ± 0.0 |
| | Ile/Val + Val/Val | 13 | 7.2 ± 3.4 |
| | **GSTA2 (Pro110Ser)** | | |
| | Pro/Pro | 23 | 7.5 ± 4.4 |
| | Pro/Ser | 2 | 8.9 ± 6.3 |
| | Ser/Ser | 0 | --- |
| | **GSTA2 (Ser112Thr)** | | |
| | Ser/Ser | 7 | 7.7 ± 5.4 |
| | Ser/Thr | 11 | 6.6 ± 4.6 |
| | Thr/Thr | 7 | 9.1 ± 3.2 |
| | Ser/Thr + Thr/Thr | 18 | 7.6 ± 4.2 |
| | **GSTA2 (Glu210Ala)** | | |
| | Glu/Glu | 21 | 8.0 ± 4.7 |
| | Glu/Ala | 4 | 5.6 ± 2.3 |
| | Ala/Ala | 0 | --- |
| **EPH** | **EPHX1 (Tyr113His + His139Arg)** | | |
| | Low activity | 12 | 7.9 ± 5.0 |
| | Medium activity | 6 | 6.3 ± 1.9 |
| | High activity | 6 | 8.0 ± 5.5 |

**Table VI.2**. Percent DNA in Tail (%Tail DNA) induced by GA in whole blood leukocytes from 25 healthy individuals considering different genotypes of genes involved in BER pathway.

| Genotypes | N | % Tail DNA ± SD |
|---|---|---|
| **XRCC1 (Gln399Arg)** | | |
| Arg/Arg | 10 | 9.6 ± 5.0 |
| Arg/Gln | 13 | 6.6 ± 3.9 |
| Gln/Gln | 2 | 4.3 ± 1.2 |
| Arg/Gln + Gln/Gln | 15 | 6.3 ± 3.7 |
| **OGG1 (Ser326Cys)** | | |
| Ser/Ser | 11 | 8.8 ± 4.0 |
| Ser/Cys | 14 | 6.6 ± 4.7 |
| Cys/Cys | 0 | ----- |
| **PARP1 (Val762Ala)** | | |
| Val/Val | 19 | 8.2 ± 3.8 |
| Val/Ala | 5 | 5.7 ± 7.1 |
| Ala/Ala | 1 | 5.5 |
| Val/Ala + Ala/Ala | 6 | 5.7 ± 6.4 |
| **PARP4 (Pro1328Thr)** | | |
| Pro/Pro | 12 | 7.7 ± 4.6 |
| Pro/Thr | 12 | 7.0 ± 4.4 |
| Thr/Thr | 1 | 13.4 |
| Pro/Thr+Thr/Thr | 13 | 7.4 ± 4.6 |
| **APEX1 (Asp148Glu)** | | |
| Glu/Glu | 8 | 7.7 ± 4.4 |
| Glu/Asp | 12 | 8.2 ± 4.9 |
| Asp/Asp | 5 | 6.0 ± 4.2 |
| Glu/Asp + Asp/Asp | 17 | 7.5 ± 4.7 |
| **MUTYH (Gln335His)** | | |
| Gln/Gln | 14 | 7.1 ± 5.3 |
| Gln/His | 7 | **6.1 ± 1.7** |
| His/His | 4 | **11.9 ± 1.8*** |
| Gln/His+His/His | 11 | 8.2 ± 3.3 |

*Note.* Asterisk indicates significant at $p<0.001$ compared to homozygous variant genotypes with heterozygous genotypes.

**Table VI.3**. Percent DNA in Tail (%Tail DNA) induced by GA in whole blood leukocytes from 25 healthy individuals considering different genotypes of genes involved in NER pathway.

| Genotypes | N | % Tail DNA ± SD |
|---|---|---|
| **ERCC2 (Lys751Gln)** | | |
| Lys/Lys | 8 | 8.2± 4.2 |
| Lys/Gln | 13 | 6.5 ± 3.5 |
| Gln/Gln | 4 | 10.0 ± 7.5 |
| Lys/Gln+ Gln/Gln | 17 | 7.3 ± 4.7 |
| **ERCC1 (Gln504Lys)** | | |
| Gln/Gln | 17 | 8.1 ± 4.9 |
| Gln/Lys | 6 | 7.3 ± 2.9 |
| Lys/Lys | 2 | 4.4 ± 5.9 |
| Gln/Lys + Lys/Lys | 8 | 6.6 ± 3.6 |
| **ERCC4 (Arg415Gln)** | | |
| Arg/Arg | 20 | 7.1 ± 4.3 |
| Arg/Gln | 3 | 10.8 ± 7.0 |
| Gln/Gln | 2 | 7.6 ± 0.7 |
| Arg/Gln + Gln/Gln | 5 | 9.5 ± 5.3 |
| **ERCC5 (His1104Asp)** | | |
| His/His | 15 | 7.9± 4.9 |
| His/Asp | 9 | 7.2 ± 4.2 |
| Asp/Asp | 1 | 5.5 |
| His/Asp + Asp/Asp | 10 | 7.1 ± 4.0 |
| **ERCC5 (Cys529Ser)** | | |
| Cys/Cys | 24 | 7.4 ± 4.5 |
| Cys/Ser | 1 | 10.9 |
| Ser/Ser | 0 | ----- |
| **ERCC6 (Arg1230Pro)** | | |
| Pro/Pro | 22 | 7.3 ± 4.5 |
| Pro/Arg | 3 | 9.6 ± 4.6 |
| Arg/Arg | 0 | ----- |
| **ERCC6 (Gln1413Arg)** | | |
| Arg/Arg | 17 | 7.6 ± 4.4 |
| Arg/Gln | 6 | 7.5 ± 5.9 |
| Gln/Gln | 2 | 7.6 ± 0.7 |
| Arg/Gln + Gln/Gln | 8 | 7.5 ± 5.0 |
| **RAD23B (Ala249Val)** | | |
| Ala/Ala | 19 | 7.1 ± 4.1 |
| Ala/Val | 6 | 9.1± 5.6 |
| Val/Val | 0 | ----- |
| **XPC (Ala499Val)** | | |
| Ala/Ala | 2 | 6.9 ± 4.3 |
| Ala/Val | 10 | 6.6 ± 6.0 |
| Val/Val | 13 | 8.4 ± 3.2 |
| Ala/Val + Val/Val | | |
| **XPC (Lys939Gln)** | | |
| Lys/Lys | 11 | 7.4 ± 5.4 |
| Lys/Gln | 12 | 8.0 ± 4.0 |
| Gln/Gln | 2 | 6.2 ± 2.6 |
| Lys/Gln + Gln/Gln | 14 | 7.8 ± 3.8 |

**Table VI.4**. Percent DNA in Tail (%Tail DNA) induced by GA in whole blood leukocytes from 25 healthy individuals considering different genotypes of genes involved in HRR pathway.

| Genotypes | N | % Tail DNA ± SD |
|---|---|---|
| **RAD51 (5'UTR)** | | |
| G/G | 6 | 10.4 ± 5.7 |
| G/T | 11 | 6.9 ± 4.7 |
| T/T | 8 | 6.4 ± 2.3 |
| G/T + T/T | 19 | 6.7 ± 3.8 |
| **NBS (Glu185Gln)** | | |
| Glu/Glu | 15 | 7.3 ± 4.6 |
| Glu/Gln | 8 | 7.7 ± 5.0 |
| Gln/Gln | 2 | 9.2 ± 1.8 |
| Glu/Gln + Gln/Gln | 10 | 8.0 ± 4.5 |
| **XRCC3 (Thr241Met)** | | |
| Thr/Thr | 6 | 5.8 ± 3.7 |
| Thr/Met | 13 | 7.9 ± 4.4 |
| Met/Met | 6 | 8.6 ± 5.7 |
| Thr/Met + Met/Met | 19 | 8.1 ± 4.7 |
| **XRCC2 (Arg188His)** | | |
| Arg/Arg | 22 | 7.0 ± 4.5 |
| Arg/His | 3 | 11.6 ± 1.5 |
| His/His | 0 | ----- |

**Table VI.5**. Percent DNA in Tail (%Tail DNA) induced by GA in whole blood leukocytes from 25 healthy individuals considering different genotypes of genes involved in NHEJ pathway.

| Genotypes | N | % Tail DNA ± SD |
|---|---|---|
| **XRCC4 Asn298Ser** | | |
| Asn/Asn | 17 | 8.4 ± 4.7 |
| Asn/Ser | 8 | 5.9 ± 3.6 |
| Ser/Ser | 0 | ------ |
| **XRCC4 Thr134Ile** | | |
| Ile/Ile | 24 | 7.8 ± 4.5 |
| Ile/Thr | 1 | 3.5 |
| Thr/Thr | 0 | ----- |
| **Lig4Thr9Ile** | | |
| Thr/Thr | 19 | 8.2 ± 4.7 |
| Thr/Ile | 6 | 5.7 ± 3.4 |
| Ile/Ile | 0 | ------ |
| **Ku80Ex21-238G>A** | | |
| G/G | 12 | 7.4 ± 4.2 |
| G/A | 8 | 6.6 ± 4.4 |
| A/A | 5 | 9.8 ± 5.5 |
| G/A+A/A | 13 | 7.8 ± 4.9 |
| **Ku80Ex21+338T>C** | | |
| T/T | 20 | 7.8 ± 4.6 |
| T/C | 4 | 6.7 ± 5.4 |
| C/C | 1 | 7.2 |
| T/C+C/C | 5 | 6.8 ± 4.6 |
| **Ku80Ex21-352C>A** | | |
| C/C | 20 | 7.8 ± 4.6 |
| C/A | 4 | 6.7 ± 5.4 |
| A/A | 1 | 7.2 |
| C/A+A/A | 5 | 6.8 ± 4.6 |
| **Ku80Ex21+466A>G** | | |
| A/A | 20 | 7.8 ± 4.7 |
| A/G | 5 | 6.8 ± 4.0 |
| G/G | 0 | ----- |

**Table VI.6.** *XRCC1* Gln399Arg and *MUTYH* Gln335His association and DNA damage

| | ***XRCC1*Gln399Arg / *MUTYH*Gln335His** | | | |
|---|---|---|---|---|
| | ArgArg+ArgGln/GlnGln+GlnHis | ArgArg+ArgGln/HisHis* | GlnGln/GlnGln+GlnHis** | Gln/Gln/His/His |
| **%Tail DNA ±SD (n)** | 7.0 ± 4.5 (19) | 11.9 ± 1.8 (4) | 4.3 ± 1.2 (2) | n.d. |

*Note*. Asterisk indicates significant difference at p=0.05 compared with ArgArg+ArgGln/GlnGln+GlnHis; Double asterisk indicates significant difference at p<0.05 compared with ArgArg+ArgGln/HisHis; n.d. non detected



**Table VI.7** *XPC* haplotypes and DNA damage

| | ***XPC*Ala499Val / *XPC*Lys939Gln** | | | | | |
|---|---|---|---|---|---|---|
| | AlaAla/LysLys | AlaVal/LysLys | AlaVal/LysGln* | ValVal/LysLys | ValVal/LysGln | ValVal/GlnGln |
| **%Tail DNA ±SD (n)** | 6.9 ± 4.3 (2) | 7.6 ± 6.2 (8) | 2.8 ± 3.7 (2) | 6.7 (1) | 9.1 ± 3.3 (10) | 6.2 ± 2.6 (2) |

*Note* Asterisk indicates significant difference at p<0.05 when compared AlaVal/LysGln with ValVal/LysGln

## 6.4. Discussion:

Acrylamide is a public health concern in terms of cancer risk assessment justifying the need of reliable toxicological biomarkers. In this context, the comet assay is actually an emerging tool to properly assess primary DNA damage either *in vitro* or *in vivo*. Using this methodology, the *in vitro* genotoxic potential of AA and GA was characterized by the % DNA in Tail in peripheral blood leukocytes from healthy donors.

Acrylamide 1-h exposure did not alter DNA damage at any concentration level tested up to 1 mM. Our results are in agreement with Baum *et al* [38], who also did not find AA genotoxicity in the comet assay with human blood cells at 1-4 h of incubation. In addition, Hansen *et al.* [39] reported an absence of AA-induced DNA damage up to 5 mM in human peripheral PBL at 2 h. However, other studies with HepG2 [40, 41], V79 cells and Caco-2 cells [9] detected significant increases in DNA damage but only at very high concentrations of AA (millimolar range). In contrast, Blasiak *et al* [42] showed significant DNA damage in isolated human lymphocytes exposed to AA at low concentrations, reinforcing the need for further evaluation of AA in human cells using different experimental conditions and protocols. In contrast to the use of isolated human lymphocytes, the present report was carried out using whole blood, an approach that can be considered more closely related to the physiological situation, since it takes into account the influence of other factors, such as red blood cells, platelets and plasma [43, 44]. Another important feature when whole blood is used is that AA reacts with haemoglobin forming Hb adducts [2], rendering AA less available to a potential cellular damaging effect.

There are convincing data showing that GA acts as the ultimate genotoxic agent in AA exposure. Our results using the comet assay revealed that GA indeed produced significant increases in terms of %Tail DNA, at concentrations starting from 250 μM. These results are in agreement with the data published by Baum *et al* [38] who observed that GA induced DNA damage for concentrations of 300 μM and highter. In addition, the results presented here are generally in accordance with other investigations that also found DNA damage for GA in the comet assay, although for higher GA concentrations (≥ 500 μM) in human lymphoblastoid TK6 cells [45] and isolated lymphocytes [39].

The results from our study also demonstrated that PBL from healthy donors respond differently *in vitro* to a given genotoxic concentration of GA. This is an important point that has not yet been fully addressed. The heterogeneity of the response observed in the comet assay using whole PBL from different donors may be in part attributed to individual genome sensitivity [46]. A plausible explanation for the interindividual variability noted herein may rely on individual genetic polymorphisms associated with detoxification and repair pathways that are likely to influence the levels of DNA damage after exposure to GA.

With respect to the detoxification polymorphisms in *GSTs* and *EPHX1* no relevant associations with GA-induced DNA damage were found. For a definitive conclusion further studies need to be performed using different endpoints of DNA damage, namely the induction of sister chromatid exchanges, which were shown to correlate with GA-DNA adducts [20].

The predominant adduct N7-GA-Gua is promutagenic since it might undergo spontaneous depurination [19, 47, 48]. The abasic site thus generated is likely to promote incorporation of deoxyadenosine during DNA replication, leading to G→T transversions [47]. DNA repair is therefore essential for the understanding of GA-induced genotoxic effects, for which a number of different primary lesions are present, including GA adducts, abasic sites and DNA breaks [20, 49]. Johansson *et al* using DNA repair deficient cell lines, suggested that the repair of the lesions induced by GA involves BER (short patch) and HRR, but not NER [49].

Concerning the BER pathway, *MUTYH* gene encodes a glycosylase involved in the repair of DNA damage resulting from the oxidation of guanine nucleotides. MUTYH protein can prevent transversions of a G:C base pair with a T:A base pair, resulting from the oxidation product of guanine that mispairs to adenine [50]. There is scarce information on the formation of reactive oxygen species by GA, although for AA some reports suggest the involvement of oxidative stress [40, 51]. Our results suggest that *MUTYH* (Gln335His) influences the genotoxicity induced by GA, since variant individuals presented higher levels of DNA damage. Further studies should be performed to clarify this point.

*XRCC1* has multiple roles in repairing DNA base damage and single-strand DNA breaks. Although XRCC1 does not have a known enzymatic activity, there is

evidence suggesting that it may act as a nucleating factor by bringing different BER components together at the site of action [52]. The importance of *XRCC1* Gln399Arg polymorphism was previously identified in occupational studies using the comet assay. Studies performed with welders [53], and healthy Japanese workers [54] associated with higher DNA damage with *XRCC1* variant allele. However, conflicting results have also been published (e.g. fruit growers exposed to pesticides) [55].

Our results suggested a significant interaction between *XRCC1* Gln399Arg and *MUTYH* Gln335His polymorphisms, as PBL from individuals with the haplotype ArgArg+ArgGln/HisHis were more prone to DNA damage than those with ArgArg+ArgGln/GlnGln+GlnHis and with GlnGln/GlnGln+GlnHis. In view of this *MUTYH* and *XRCC1*, seem to be involved in the genotoxicity induced by GA. Overall, our results emphasize that the BER pathway may be operative in the repair of lesions generated by GA. However, as a consequence of sample size, further confirmations need to be made in a larger population.

Data presented here also suggest an association of global genome NER pathway, specifically XPC, and GA-induced DNA damage. XPC is involved in DNA damage recognition, and in DNA repair initiation. The binding of XPC to damaged DNA is the rate-limiting step for NER [56]. Several epidemiological studies have been carried out to evaluate the association of XPC polymorphisms with cancer risk at different organ sites and in diverse populations (reviewed in [56]). Further, a study performed by Wang *et al* [57] suggested that the polymorphisms of XPC genes might modulate the genotoxicity by PAH in coke oven workers. Our results suggest that XPC Ala499Val/Lys939Gln diplotype, might be associated with DNA damage induced by GA.

Overall, this study shows that GA, but not AA, increases DNA damage as measured by the comet assay. Using this methodology, the interindividual variation observed in terms of GA-induced genotoxicity might be associated with polymorphic genes involved in BER and NER pathways. Further studies should be performed to reinforce these findings, including a larger number of individuals with different genotypes. Further, functional studies are required in order to understand the underlying mechanisms of the variability in the GA-induced DNA damage.

## 6.5. References


1. IARC: **Acrylamide**. In: *Acrylamide In IARC monographs on the evaluation of carcinogenic risk to humans Volume 60*, edn.: International Agency for Research on Cancer, Lyon; 1994: 389-433.

2. Carere A: **Genotoxicity and carcinogenicity of acrylamide: a critical review**. *AnnIstSuperSanita* 2006, **42**(2):144-155.

3. Friedman M: **Chemistry, biochemistry, and safety of acrylamide. A review**. *JAgricFood Chem* 2003, **51**(16):4504-4526.

4. Parzefall W: **Minireview on the toxicity of dietary acrylamide**. *Food ChemToxicol* 2008, **46**(4):1360-1364.

5. Ruden C: **Acrylamide and cancer risk--expert risk assessments and the public debate**. *Food ChemToxicol* 2004, **42**(3):335-349.

6. Exon JH: **A review of the toxicology of acrylamide**. *Journal of toxicology and environmental health Part B, Critical reviews* 2006, **9**(5):397-412.

7. Rice JM: **The carcinogenicity of acrylamide**. *MutatRes* 2005, **580**(1-2):3-20.

8. Stadler RH, Blank I, Varga N, Robert F, Hau J, Guy PA, Robert MC, Riediker S: **Acrylamide from Maillard reaction products**. *Nature* 2002, **419**(6906):449-450.

9. Puppel N, Tjaden Z, Fueller F, Marko D: **DNA strand breaking capacity of acrylamide and glycidamide in mammalian cells**. *Mutation research* 2005, **580**(1-2):71-80.

10. Dybing E, Sanner T: **Risk assessment of acrylamide in foods**. *ToxicolSci* 2003, **75**(1):7-15.

11. Tareke E, Rydberg P, Karlsson P, Eriksson S, Tornqvist M: **Analysis of acrylamide, a carcinogen formed in heated foodstuffs**. *Journal of agricultural and food chemistry* 2002, **50**(17):4998-5006.

12. Konings EJ, Baars AJ, van Klaveren JD, Spanjer MC, Rensen PM, Hiemstra M, van Kooij JA, Peters PW: **Acrylamide exposure from foods of the Dutch population and an assessment of the consequent risks**. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 2003, **41**(11):1569-1579.

13. Abou-Donia MB, Ibrahim SM, Corcoran JJ, Lack L, Friedman MA, Lapadula DM: **Neurotoxicity of glycidamide, an acrylamide metabolite, following intraperitoneal injections in rats**. *Journal of toxicology and environmental health* 1993, **39**(4):447-464.

14. Kirman CR, Gargas ML, Deskin R, Tonner-Navarro L, Andersen ME: **A physiologically based pharmacokinetic model for acrylamide and its metabolite, glycidamide, in the rat**. *Journal of toxicology and environmental health Part A* 2003, **66**(3):253-274.

15. Ghanayem BI, McDaniel LP, Churchwell MI, Twaddle NC, Snyder R, Fennell TR, Doerge DR: **Role of CYP2E1 in the epoxidation of acrylamide to glycidamide and formation of DNA and hemoglobin adducts**. *Toxicological sciences : an official journal of the Society of Toxicology* 2005, **88**(2):311-318.

16. Ghanayem BI, Witt KL, Kissling GE, Tice RR, Recio L: **Absence of acrylamide-induced genotoxicity in CYP2E1-null mice: evidence consistent with a glycidamide-mediated effect**. *Mutation research* 2005, **578**(1-2):284-297.

17. Glatt H, Schneider H, Liu Y: **V79-hCYP2E1-hSULT1A1, a cell line for the sensitive detection of genotoxic effects induced by carbohydrate pyrolysis products and other food-borne chemicals**. *MutatRes* 2005, **580**(1-2):41-52.

18. Doerge DR, Young JF, McDaniel LP, Twaddle NC, Churchwell MI: **Toxicokinetics of acrylamide and glycidamide in Fischer 344 rats**. *Toxicology and applied pharmacology* 2005, **208**(3):199-209.

19. Gamboa da Costa G, Churchwell MI, Hamilton LP, Von Tungeln LS, Beland FA, Marques MM, Doerge DR: **DNA adduct formation from acrylamide via conversion to glycidamide in adult and neonatal mice**. *Chemical research in toxicology* 2003, **16**(10):1328-1337.

20. Martins C, Oliveira NG, Pingarilho M, Gamboa da CG, Martins V, Marques MM, Beland FA, Churchwell MI, Doerge DR, Rueff J *et al*: **Cytogenetic damage induced by acrylamide and glycidamide in mammalian cells: correlation with specific glycidamide-DNA adducts**. *ToxicolSci* 2007, **95**(2):383-390.

21. Burley VJ, Greenwood DC, Hepworth SJ, Fraser LK, de Kok TM, van Breda SG, Kyrtopoulos SA, Botsivali M, Kleinjans J, McKinney PA *et al*: **Dietary acrylamide intake and risk of breast cancer in the UK women's cohort**. *BrJCancer* 2010, **103**(11):1749-1754.

22. Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA: **A prospective study of dietary acrylamide intake and the risk of endometrial, ovarian, and breast cancer**. *Cancer EpidemiolBiomarkers Prev* 2007, **16**(11):2304-2313.

23. Olesen PT, Olsen A, Frandsen H, Frederiksen K, Overvad K, Tjonneland A: **Acrylamide exposure and incidence of breast cancer among postmenopausal women in the Danish Diet, Cancer and Health Study**. *IntJCancer* 2008, **122**(9):2094-2100.

24. Pelucchi C, Galeone C, Levi F, Negri E, Franceschi S, Talamini R, Bosetti C, Giacosa A, La Vecchia C: **Dietary acrylamide and human cancer**. *International journal of cancer Journal international du cancer* 2006, **118**(2):467-471.

25. Wilson KM, Mucci LA, Cho E, Hunter DJ, Chen WY, Willett WC: **Dietary acrylamide intake and risk of premenopausal breast cancer**. *AmJEpidemiol* 2009, **169**(8):954-961.

26. Costa S, Coelho P, Costa C, Silva S, Mayan O, Santos LS, Gaspar J, Teixeira JP: **Genotoxic damage in pathology anatomy laboratory workers exposed to formaldehyde**. *Toxicology* 2008, **252**(1-3):40-48.

27. Synowiec E, Stefanska J, Morawiec Z, Blasiak J, Wozniak K: **Association between DNA damage, DNA repair genes variability and clinical characteristics in breast cancer patients**. *Mutation research* 2008, **648**(1-2):65-72.

28. Collins AR, Oscoz AA, Brunborg G, Gaivao I, Giovannelli L, Kruszewski M, Smith CC, Stetina R: **The comet assay: topical issues**. *Mutagenesis* 2008, **23**(3):143-151.

29. Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR, Escobar P, Honma M, Kumaravel TS, Nakajima M *et al*: **Fourth International Workgroup on Genotoxicity testing: results of the in vivo Comet assay workgroup**. *Mutation research* 2007, **627**(1):31-35.

30. Singh NP, McCoy MT, Tice RR, Schneider EL: **A simple technique for quantitation of low levels of DNA damage in individual cells**. *Experimental cell research* 1988, **175**(1):184-191.

31. Busto N, Garcia B, Leal JM, Gaspar JF, Martins C, Boggioni A, Secco F: **ACMA (9-amino-6-chloro-2-methoxy acridine) forms three complexes in the presence of DNA**. *Physical chemistry chemical physics : PCCP* 2011, **13**(43):19534-19545.

32. Fracasso ME, Doria D, Franceschetti P, Perbellini L, Romeo L: **DNA damage and repair capacity by comet assay in lymphocytes of white-collar active smokers and passive smokers (non- and ex-smokers) at workplace**. *Toxicology letters* 2006, **167**(2):131-141.

33. Lin DX, Tang YM, Peng Q, Lu SX, Ambrosone CB, Kadlubar FF: **Susceptibility to esophageal cancer and genetic polymorphisms in glutathione S-transferases T1, P1, and M1 and cytochrome P450 2E1**. *Cancer EpidemiolBiomarkers Prev* 1998, **7**(11):1013-1018.

34. Bastos HN, Antao MR, Silva SN, Azevedo AP, Manita I, Teixeira V, Pina JE, Gil OM, Ferreira TC, Limbert E *et al*: **Association of polymorphisms in genes of the homologous recombination DNA repair pathway and thyroid cancer risk**. *Thyroid* 2009, **19**(10):1067-1075.

35. Conde J, Silva SN, Azevedo AP, Teixeira V, Pina JE, Rueff J, Gaspar JF: **Association of common variants in mismatch repair genes and breast cancer susceptibility: a multigene study**. *BMCCancer* 2009, **9**:344.

36. Gomes BC, Silva SN, Azevedo AP, Manita I, Gil OM, Ferreira TC, Limbert E, Rueff J, Gaspar JF: **The role of common variants of non-homologous end-joining repair genes XRCC4, LIG4 and Ku80 in thyroid cancer risk**. *OncolRep* 2010, **24**(4):1079-1085.

37. Silva SN, Azevedo AP, Teixeira V, Pina JE, Rueff J, Gaspar JF: **The role of GSTA2 polymorphisms and haplotypes in breast cancer susceptibility: a case-control study in the Portuguese population**. *OncolRep* 2009, **22**(3):593-598.

38. Baum M, Fauth E, Fritzen S, Herrmann A, Mertes P, Merz K, Rudolphi M, Zankl H, Eisenbrand G: **Acrylamide and glycidamide: genotoxic effects in V79-cells and human blood**. *Mutation research* 2005, **580**(1-2):61-69.

39. Hansen SH, Olsen AK, Soderlund EJ, Brunborg G: **In vitro investigations of glycidamide-induced DNA lesions in mouse male germ cells and in mouse and human lymphocytes**. *Mutation research* 2010, **696**(1):55-61.

40. Cao J, Liu Y, Jia L, Jiang LP, Geng CY, Yao XF, Kong Y, Jiang BN, Zhong LF: **Curcumin attenuates acrylamide-induced cytotoxicity and genotoxicity in HepG2 cells by ROS scavenging**. *Journal of agricultural and food chemistry* 2008, **56**(24):12059-12063.

41. Jiang L, Cao J, An Y, Geng C, Qu S, Zhong L: **Genotoxicity of acrylamide in human hepatoma G2 (HepG2) cells**. *Toxicology in vitro : an international journal published in association with BIBRA* 2007, **21**(8):1486-1492.

42. Blasiak J, Gloc E, Wozniak K, Czechowska A: **Genotoxicity of acrylamide in human lymphocytes**. *Chemico-biological interactions* 2004, **149**(2-3):137-149.

43. Ramos DL, Gaspar JF, Pingarilho M, Gil OM, Fernandes AS, Rueff J, Oliveira NG: **Genotoxic effects of doxorubicin in cultured human lymphocytes with different glutathione S-transferase genotypes**. *MutatRes* 2011, **724**(1-2):28-34.

44. Rueff J, Chiapella C, Chipman JK, Darroudi F, Silva ID, Duverger-van BM, Fonti E, Glatt HR, Isern P, Laires A *et al*: **Development and validation of alternative metabolic systems for mutagenicity testing in short-term assays**. *MutatRes* 1996, **353**(1-2):151-176.

45. Koyama N, Sakamoto H, Sakuraba M, Koizumi T, Takashima Y, Hayashi M, Matsufuji H, Yamagata K, Masuda S, Kinae N *et al*: **Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells**. *Mutation research* 2006, **603**(2):151-158.

46. Kopjar N, Zeljezic D, Garaj-Vrhovac V: **Evaluation of DNA damage in white blood cells of healthy human volunteers using the alkaline comet assay and the chromosome aberration test**. *Acta BiochimPol* 2006, **53**(2):321-336.

47. Besaratinia A, Pfeifer GP: **Genotoxicity of acrylamide and glycidamide**. *JNatlCancer Inst* 2004, **96**(13):1023-1029.

48. Segerback D, Calleman CJ, Schroeder JL, Costa LG, Faustman EM: **Formation of N-7-(2-carbamoyl-2-hydroxyethyl)guanine in DNA of the mouse and the rat following intraperitoneal administration of [14C]acrylamide**. *Carcinogenesis* 1995, **16**(5):1161-1165.

49. Johansson F, Lundell T, Rydberg P, Erixon K, Jenssen D: **Mutagenicity and DNA repair of glycidamide-induced adducts in mammalian cells**. *Mutation research* 2005, **580**(1-2):81-89.

50. Nielsen M, van Steenbergen LN, Jones N, Vogt S, Vasen HF, Morreau H, Aretz S, Sampson JR, Dekkers OM, Janssen-Heijnen ML *et al*: **Survival of MUTYH-associated polyposis patients with colorectal cancer and matched control colorectal cancer patients**. *Journal of the National Cancer Institute* 2010, **102**(22):1724-1730.

51. Zeiger E, Recio L, Fennell TR, Haseman JK, Snyder RW, Friedman M: **Investigation of the low-dose response in the in vivo induction of micronuclei and adducts by acrylamide**. *ToxicolSci* 2009, **107**(1):247-257.

52. Silva SN, Moita R, Azevedo AP, Gouveia R, Manita I, Pina JE, Rueff J, Gaspar J: **Menopausal age and XRCC1 gene polymorphisms: role in breast cancer risk**. *Cancer detection and prevention* 2007, **31**(4):303-309.

53. Iarmarcovai G, Bonassi S, Botta A, Baan RA, Orsiere T: **Genetic polymorphisms and micronucleus formation: a review of the literature**. *MutatRes* 2008, **658**(3):215-233.

54. Weng H, Weng Z, Lu Y, Nakayama K, Morimoto K: **Effects of cigarette smoking, XRCC1 genetic polymorphisms, and age on basal DNA damage in human blood mononuclear cells**. *Mutation research* 2009, **679**(1-2):59-64.

55. Wong RH, Chang SY, Ho SW, Huang PL, Liu YJ, Chen YC, Yeh YH, Lee HS: **Polymorphisms in metabolic GSTP1 and DNA-repair XRCC1 genes with an increased risk of DNA damage in pesticide-exposed fruit growers**. *Mutation research* 2008, **654**(2):168-175.

56. Qiu L, Wang Z, Shi X: **Associations between XPC polymorphisms and risk of cancers: A meta-analysis**. *Eur J Cancer* 2008, **44**(15):2241-2253.

57. Wang Y, Yang H, Li L, Wang H, Zhang C, Yin G, Zhu B: **Association between CYP2E1 genetic polymorphisms and lung cancer risk: a meta-analysis**. *Eur J Cancer* 2010, **46**(4):758-764.

# Chapter 7

## Concluding remarks and future prospects

## 7.1. Concluding remarks and future prospects

Acrylamide is a well-known industrial chemical classified as probable human carcinogen by IARC since 1994 [1] presented in stuff foods [2]. This carcinogen can be formed by Maillard reaction between primarily the amino acid asparagine and reducing sugars in food processing upon heating [3, 4]. Furthermore, AA can be found in commonly consumed foods and beverages, such as processed cereals, French fries, potato chips and coffee [2]. The average daily intake of AA was estimated to be about 0.5-1.0 µg/Kg bw in adults and up to 2-fold in 13 year-old children with a normal western diet [2]. This has raised concerns about the positive health risks of AA for the general population, even more because AA is a known rodent carcinogen [5]. Furthermore, the association of the increased risk of human cancer with AA dietary consumption is still a matter of discussion [5, 6].

AA can be metabolized by cytochrome P450 (CYP2E1) to the genotoxic epoxide glycidamide (GA), the ultimate genotoxin. However, the mechanisms of genotoxicity of AA- and GA- dependent in mammalian cells are not yet fully understood. In view of this, the evaluation of DNA damage induced by these two compounds is of major importance. With this purpose, we resorted to the use of distinct assays: chromosomal aberrations (CAs), sister chromatid exchange (SCEs), quantification of DNA adducts and comet assay in V79 Chinese hamster cells and in human lymphocytes.

V79 cells are a well established mammalian cell line widely used in cytotoxicity and genotoxicity studies. These cells are very important in present study as they do not express detectable levels of CYP2E1, the recognized cytochrome P450, responsible for AA epoxidation [7, 8]. These cells revealed to be an adequate model to properly address the mechanisms of genotoxicity by AA and GA. However, the use of human cells is obviously more suitable for human risk assessment, and also provides the possibility to address inter-individual variation in terms of DNA damage and the association with individual susceptibility. The use of human cells from whole blood samples was chosen in this work since it is considered to be more closely related to the physiological situation than isolated lymphocytes, including the influence of others factors, such as red blood cells, platelets and plasma [9].

In view of that, the cytotoxicity of AA and GA in V79 cells, evaluated by the MTT reduction assay, was performed. The results showed that both AA and GA induced dose-dependent cell death, being GA clearly more cytotoxic than AA for all the concentrations studied, which is in agreement with data reported by others groups, using different cell survival end points [10, 11].

The clastogenicity of these compounds were then analyzed by CAs assay in V79 cells. The results suggested that both AA and GA induced CAs, especially chromatid breaks and gaps. AA and GA increased the % ACEG mainly for the higher concentration studied (1000 and 2000 μM, for GA and AA, respectively).

The genotoxicity of AA and GA were also analyzed by SCEs assay in V79 cells and in stimulated human lymphocytes. The results concerning SCE formation in V79 cells clearly showed that GA consistently induced SCEs for concentrations above 10 μM, increasing the background levels of SCEs by about 10-fold to levels of about 60 SCE/cell for the highest concentration tested. On the other hand, for AA-exposed cultures, a significant increase in SCE/cell was observed only at a very high concentration level (2000 μM). The induction of SCE in lymphocytes revealed results that are in agreement with the previous one obtained in V79 cells, since the induction of SCEs by AA was slight and specially associated with the very high and cytotoxic concentration of 2000 μM and that GA markedly induced SCE in a dose-response manner (up to 750 μM). These results reinforce the knowledge that GA is far more genotoxic than AA and also highlight the high sensitivity of SCE to be used as a toxicological biomarker in an AA exposure scenario.

The levels of N7-GA-Gua and N3-GA-Ade were also measured in V79 cells and in stimulated lymphocytes exposed to AA and GA. Data showed that AA exposure in V79 cells lead to very low levels of N7-GA-Gua, which were only observed for concentrations higher than 1000 μM. These low levels of N7-GA-Gua stemming from AA exposure might be related to either residual metabolism of AA in V79 cells or to a small extent of spontaneous nonenzymatic oxidation to GA, since V79 cells are essentially devoid of CYP2E1. On the other hand, GA showed to be a potent inducer of N7-GA-Gua with linear dose-response dependence. The detection of this adducts was observed for doses as low as 1 μM and 10 μM of GA, respectively in V79 cells and in lymphocytes. The second more frequent adduct, N3-GA-Ade was only detected at very

high GA concentrations precluding the interest of its quantification in human dietary studies. It is recognized that compounds that form DNA adducts are also strong SCEs inducers. Our results showed, in fact, a very strong correlation between the levels of N7-GA-Gua and SCE/cell for both cell types. This finding is very important in terms of the understanding of the mechanisms involved in DNA damage signaling and repair. These results suggested that the metabolism of AA to GA and the ensuing formation of depurinating DNA lesions [12, 13] are responsible for SCE induction.

In view of these showed clearly differences between AA and GA genotoxicity, it seemed important to clarify the genotoxicity mechanisms induced by AA *per se.* The modulation of reduced glutathione (GSH) status could give additional insight into this matter, as GSH is a key factor for mammalian cells homeostasis, with diverse functions that include, among others, the conjugation of eletrophilic compounds, including AA and GA, and the detoxification of products generated by oxidative stress. For this purpose the cytotoxicity was analyzed by the MTT assay and clastogenicity evaluated by CAs. This study evaluated the effect of GSH modulators, including the evaluation of the effect of buthionine sulfoximine (BSO), of GSH-monoethyl ester (GSH-EE) and also of GSH endogenously added to culture medium in V79 cells. BSO treatment alone was associated with relatively low toxicity leading to a slight decrease in cell viability and a moderate increase in the frequency of ACEG. In order to evaluate the potential protective effect of GSH towards AA toxicity, the intracellular GSH enrichment was performed by pre-treatment with GSH-EE. The results obtained fail to show the protective effect of GSH-EE. Co-treatment with GSH exogenously added to the culture medium revealed a protective effect either in terms of cytotoxicity or clastogenicity induced by AA. This effect could be a consequence of the spontaneous conjugation of AA with GSH in the extracellular medium. In this sense, the evaluation of the potential conjugation of AA with GSH in the extracellular medium was performed. With this purpose, we studied in a set of cell free experiments the intensity of fluorescence of the conjugate GSH-monochorobimane (MCB), a fluorescent probe for GSH, after incubation of AA with GSH. These results showed that spontaneous conjugation of AA is favored when AA concentrations are higher than GSH concentrations and that spontaneous conjugation is clearly time-dependent (1-h incubation decreased the percentage of free GSH when compared with a 24-h incubation period). The results

presented here reinforce the role of GSH in the modulation of the cytotoxic and clastogenic effects induced by AA.

The "single cell gel electrophoresis" or comet assay was also performed to study the genotoxicity of AA and GA in human cells. We compared the DNA damage potency of AA and its metabolite GA in the comet assay using human leukocytes (whole blood samples) and the results revealed that AA was not genotoxic up to 1000 µM, while GA showed to significantly increase % Tail DNA at concentrations starting from 250 µM.

Both SCE and comet assay showed some extent of inter-individual variability that could be related with different polymorphisms in detoxification and DNA repair genes. In view of this, the different donors were genotyped for detoxification (*GSTM1*, *GSTT1*, *GSTP1*, *GSTA2* and *EPHX1)* and DNA repair genes involved in BER (*APEX1*, *MUTYH, OGG1*, *PARP1*, *PARP4*, and XRCC1), NER (*ERCC1*, *ERCC2*, *ERCC4*, *ERCC5*, *ERCC6*, *RAD23B* and *XPC*), HRR (*NBS*, *RAD51*, *XRCC2* and *XRCC3*) and NHEJ (*Ku80, Lig4* and *XRCC4*) pathways. These polymorphic genes were analyzed in order to find out possible associations between genotypes and GA-induced SCE frequency and % Tail DNA.

By combining DNA damage, assessed by SCE assay, in GA-treated lymphocytes and polymorphisms data, associations between the induction of SCEs and *GSTP1* (Ile105Val) and *GSTA2* (Glu210Ala) genotypes are suggested, but not the other polymorphic genes associated with DNA repair pathways. Moreover, our results suggested associations between DNA damage, assessed by the alkaline comet assay, and polymorphisms of BER (*MUTYH* Gln335His and *XRCC1* Gln399Arg) and NER (*XPC* Ala499Val) genes, either alone or in combination. Further studies should be performed in order to evaluate other polymorphisms and different combinations of polymorphisms. These future studies necessarily need larger population samples and will be important to reinforce these findings and understand the underlying mechanisms of variability in GA-induced DNA damage.

The overall results showed that GA is far more genotoxic than AA in all the endpoints studied and in both types of cell models used. All biomarkers developed in the framework of this study revealed to be adequate to understand the mechanisms triggered upon AA or GA exposure. Nonetheless, the use of specific N7-GA-Gua a GA-

DNA adduct revealed to be of utmost importance in terms of toxicological significance in view of the high sensitivity, being much relevant its quantification in cultured cells exposed to GA at concentrations as low as 1 μM. Taken together the results from this thesis strongly point out that GA, the epoxide metabolite of AA is the responsible for the genotoxic effects of AA.

In the future it would be important to validate these results with a group of young Portuguese individuals, taking into account the amount of foodstuff consumed containing AA. This study is already being performed by us and shall evaluate the results obtained in a food frequency questionnaire and the basal levels of DNA damage in this group using the cytogenetic biomarkers (SCEs, CAs, comet assay and DNA-adducts) validated in this work.

## 7.2. References


1. IARC: **Acrylamide**. In: *Acrylamide In IARC monographs on the evaluation of carcinogenic risk to humans Volume 60*, edn.: International Agency for Research on Cancer, Lyon; 1994: 389-433.

2. Dybing E, Sanner T: **Risk assessment of acrylamide in foods**. *ToxicolSci* 2003, **75**(1):7-15.

3. Stadler RH, Blank I, Varga N, Robert F, Hau J, Guy PA, Robert MC, Riediker S: **Acrylamide from Maillard reaction products**. *Nature* 2002, **419**(6906):449-450.

4. Friedman M: **Chemistry, biochemistry, and safety of acrylamide. A review**. *JAgricFood Chem* 2003, **51**(16):4504-4526.

5. Rice JM: **The carcinogenicity of acrylamide**. *MutatRes* 2005, **580**(1-2):3-20.

6. Parzefall W: **Minireview on the toxicity of dietary acrylamide**. *Food ChemToxicol* 2008, **46**(4):1360-1364.

7. Doehmer J, Dogra S, Friedberg T, Monier S, Adesnik M, Glatt H, Oesch F: **Stable expression of rat cytochrome P-450IIB1 cDNA in Chinese hamster cells (V79) and metabolic activation of aflatoxin B1**. *Proceedings of the National Academy of Sciences of the United States of America* 1988, **85**(16):5769-5773.

8. Glatt H, Schneider H, Liu Y: **V79-hCYP2E1-hSULT1A1, a cell line for the sensitive detection of genotoxic effects induced by carbohydrate pyrolysis products and other food-borne chemicals**. *MutatRes* 2005, **580**(1-2):41-52.

9. Rueff J, Chiapella C, Chipman JK, Darroudi F, Silva ID, Duverger-van BM, Fonti E, Glatt HR, Isern P, Laires A *et al*: **Development and validation of alternative metabolic systems for mutagenicity testing in short-term assays**. *MutatRes* 1996, **353**(1-2):151-176.

10. Baum M, Fauth E, Fritzen S, Herrmann A, Mertes P, Merz K, Rudolphi M, Zankl H, Eisenbrand G: **Acrylamide and glycidamide: genotoxic effects in V79-cells and human blood**. *Mutation research* 2005, **580**(1-2):61-69.

11. Koyama N, Sakamoto H, Sakuraba M, Koizumi T, Takashima Y, Hayashi M, Matsufuji H, Yamagata K, Masuda S, Kinae N *et al*: **Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells**. *Mutation research* 2006, **603**(2):151-158.

12. Ghanayem BI, McDaniel LP, Churchwell MI, Twaddle NC, Snyder R, Fennell TR, Doerge DR: **Role of CYP2E1 in the epoxidation of acrylamide to glycidamide and formation of DNA and hemoglobin adducts**. *Toxicological sciences : an official journal of the Society of Toxicology* 2005, **88**(2):311-318.

13. Ghanayem BI, Witt KL, Kissling GE, Tice RR, Recio L: **Absence of acrylamide-induced genotoxicity in CYP2E1-null mice: evidence consistent with a glycidamide-mediated effect**. *Mutation research* 2005, **578**(1-2):284-297.


.

# Supplement

**Resumo**

A acrilamida (AA) é um composto químico, utilizado principalmente como agente floculante no processo de depuração de água potável e tratamento de águas residuais urbanas e industriais, e como agente de controlo de fluxo em operações em poços de petróleo. Por outro lado, a AA é utilizada em engenharia civil, em fundações, como constituinte da argamassa utilizada na construção e reparação de esgotos, túneis e barragens. A AA é ainda utilizada na estabilização de solos, síntese de corantes, produção de materiais de embalagem, co-polímeros para lentes de contacto, cosméticos, e em muitos laboratórios de biologia molecular e engenharia genética na preparação de géis de electroforese.

A AA foi classificada como potencial carcinogéneo para os humanos, pela IARC *(International Agency for Research on Cancer)*, com base em resultados obtidos em estudos com animais. Estudos de carcinogenicidade em ratos mostraram que a AA aumenta o número de tumores em diferentes órgãos, incluindo a glândula mamária. Foi também demostrado que a AA é neurotóxica em humanos e em experiências com animais, e que apresenta propriedades mutagénicas em células somáticas e germinais. A AA absorvida pode ser oxidada ao seu metabolito epóxido glicidamida (GA) através do citocromo P450 2E1, podendo ambas, AA e GA, ser conjugadas com o glutationo reduzido (GSH), originando conjugados de ácido mercaptúrico, que serão eliminados pela urina. A GA, por sua vez, pode também ser conjugada com a epóxide hidrolase, originando a gliceramida. Tanto a AA como a GA podem ligar-se a macromoléculas, tais como a hemoglobina e o DNA originando aductos específicos. Estes aductos podem ser usados como biomarcadores de dose efetiva de exposição à AA e GA.

Durante a década de 90 pensava-se que o homem estava exposto à AA apenas através da exposição ocupacional, e também apenas em pequenas quantidades através do consumo de água potável refinada com poliacrilamida, ou ainda pela inalação do fumo de tabaco. No entanto, alguns estudos verificaram a existência de níveis elevados de aductos de AA-hemoglobina em indivíduos não expostos, o que provocou uma forte preocupação em termos de risco para a saúde humana, principalmente porque foram descobertos níveis relativamente elevados de AA em diversos alimentos ricos em amido quando sujeitos a elevado tratamento térmico. Os referidos produtos alimentares, além

de serem consumidos em larga escala, são especialmente consumidos por crianças e jovens, sendo que nos mesmos se incluem por exemplo as batatas fritas, "snacks", cereais de pequeno-almoço, bolachas e café, tendo sido estimado que a dose total diária de AA ingerida por adulto tendo como base uma dieta ocidental normal, se encontra na ordem dos 1 μg/Kg por peso corporal, podendo ser 2 a 3 vezes superior em crianças e adolescentes.

Desde 2002 as investigações sobre a presença de AA nos alimentos, e as possíveis consequências para a saúde pública têm-se sucedido e vários progressos têm sido alcançados. No entanto, até à data os resultados são escassos tendo em conta os mecanismos de genotoxicidade da AA e GA. Por esse motivo, o objectivo desta tese centra-se na avaliação das consequências genéticas da exposição à AA e GA, recorrendo ao uso de linhas celulares e de linfócitos humanos.

Neste estudo procedeu-se inicialmente à avaliação da citotoxicidade e das lesões de DNA induzidas pela AA e GA em células de hamster Chinês V79, uma linha celular deficiente em CYP2E1, de modo a prevenir a conversão de AA a GA (Capítulo3). Para avaliar a citoxicidade recorreu-se ao ensaio do MTT para concentrações compreendidas entre 0.1 e 10 mM. A AA mostrou ser menos citotóxica do que a GA para todas as concentrações testadas, apresentando baixos níveis de sobrevivência (<5%) para concentrações elevadas de GA (≥ 4mM) e de AA (10 mM).

De modo a avaliar a genotoxicidade e a clastogenicidade da AA e GA em células de mamífero, recorreu-se à utilização de dois testes citogenéticos diferentes: o teste das aberrações cromossómicas (CAs), e o teste da troca de cromátides irmãs (SCEs). Os resultados obtidos no teste das CAs em células V79, mostraram claramente que tanto a AA como a GA, são compostos clastogénicos induzindo a formação de aberrações cromossómicas, com uma dependência dose-resposta, especialmente observada para as concentrações mais elevadas (1000 μM e 2000 μM). No entanto, para concentrações equimolares a GA mostrou ser mais clastogénica do que a AA. Através dos resultados obtidos no teste da troca de cromátides irmãs, na mesma linha celular, pode-se afirmar que a GA é um potente indutor de SCEs para concentrações igual ou superiores a 10 μM, com uma clara relação dose-resposta, aumentando a frequência basal de troca de cromátides irmãs em cerca de 10 vezes, para níveis de aproximadamente 60 SCE/célula para a concentração mais elevada (1000 μM). Em

contrapartida, a AA demonstrou ser um fraco indutor de SCEs, observando-se apenas um aumento no nível de lesão para a concentração mais elevada (2000 μM). Os resultados no geral, para todas as doses testadas, mostraram que a GA é mais citotóxica e clastogénica do que a AA em células V79. Este estudo demonstrou também a elevada sensibilidade do ensaio da troca de cromátides irmãs, de modo a poder ser utilizado como biomarcador toxicológico no caso de um cenário de exposição à AA.

No âmbito desta tese efetuou-se também a quantificação de aductos específicos de DNA, nomeadamente N7-(2-carbamoil-2-hidroxietil)guanina (N7-GA-Gua) e N3-(2-carbamoil-2-hidroxietil)adenina (N3-GA-Ade) em células V79. Os resultados mostraram que a exposição à AA origina níveis muito baixos de aductos N7-GA-Gua, os quais foram apenas observados para concentrações superiores a 1000 μM. Estes baixos níveis de aductos que são provenientes da exposição à AA devem estar relacionados com a oxidação não enzimática espontânea da AA a GA, uma vez que as células V79 são deficientes em CYP2E1. No entanto, a GA mostrou ser um potente indutor de N7-GA-Gua, apresentando uma dose-resposta linear, sendo a detecção deste aducto observada para concentrações tão baixas como 1 μM. O segundo aducto mais frequente, N3-GA-Ade foi apenas detectado para concentrações muito elevadas de GA, limitando o seu interesse em estudos sobre dieta humana. Adicionalmente, observou-se que as concentrações de GA e os níveis de N7-GA-Gua apresentam uma relação dose-resposta linear. Obteve-se também uma óptima correlação entre os níveis de N7-GA-Gua e o nível de SCE/célula ($r=0.987$; $p=1.25x10^{-12}$).

Durante este trabalho procurou-se também compreender os mecanismos da genotoxicidade induzida pela AA. Para tal estudaram-se os mecanismos de modulação do glutationo reduzido (GSH), o qual possui vários papéis importantes nas células de mamífero, incluindo a conjugação com compostos electrofílicos e a destoxificação de produtos obtidos por stress oxidativo. Deste modo, foram efetuados ensaios onde se averiguou o efeito da deplecção intracelular de GSH (pré-incubação com butionina sulfoximina, BSO), o efeito do enriquecimento intracelular com GSH (pré-incubação com GSH-monoetil estér, GSH-EE) e o papel do GSH adicionado extracelularmente (através da co-incubação com GSH) em células V79 (Capítulo 4). Com este objectivo, avaliaram-se diferentes parâmetros, tais como a citotoxicidade (ensaio do MTT) e a clastogenicidade (ensaio das ACs). Nos ensaios das células V79 expostas a AA e com pré-incubação com BSO, verificou-se um decréscimo na viabilidade celular e um

aumento na fequência das aberrações cromossómicas excluindo *gaps* (ACEG). Por outro lado, através da pré-incubação com GSH-EE não se verificaram alterações significativas na percentagem de sobrevivência das células V79 expostas à AA, nem na frequência de ACEG. Por fim, no co-tratamento com GSH adicionado exogenamente ao meio de cultura, verificou-se um efeito protectivo tanto em termos de viabilidade celular quer em termos de clastogenicidade induzidas pela AA. Este efeito pode ser consequência da conjugação expontânea da AA com GSH no meio extracelular. Deste modo, foi posteriormente avaliado o potencial de conjugação da AA com o GSH com base no teste da fluorescência do monoclorobimano (MCB), uma sonda fluorescente que se liga ao GSH. Os resultados mostraram que a conjugação expontênea da AA é favorecida quando existem concentrações superiores de AA em relação ao GSH e que esta conjugação expontânea é claramente dependente do tempo de exposição (1h de exposição diminuiu a percentagem de GSH livre quando comparado com o período de 24h de exposição). Globalmente, os resultados obtidos reforçaram o papel do GSH na modulação da citotoxicidade e clastogenicidade induzidas pela AA.

Apesar do uso das células V79 ser adequado como modelo para inferir sobre a genotoxicidade induzida pela AA e GA, o passo seguinte deste trabalho envolveu o estudo em células humanas. A utilização deste tipo de células é obviamente mais adequado para a avaliação de risco, permitindo também a possibilidade de investigar a variabilidade individual em termos de lesão de DNA, e a sua correlação com a susceptibilidade de cada individuo. Para tal recorreu-se à colheita de sangue periférico de dadores saudáveis, não sujeitos a medicação e não fumadores. Os dadores foram devidamente informados sobre o âmbito do estudo, e um consentimento informado foi assinado pelos mesmos, antes de se ter procedido ao início dos trabalhos. Cumprirá também salientar o facto de ter sido obtida uma aprovação do estudo pelo Comité de Ética da Faculdade de Ciências Médicas da Universidade Nova de Lisboa. No referido estudo recorreu-se ao uso de células humanas provenientes de sangue total, uma vez que é considerado ser mais aproximado da situação fisiológica do que o uso de linfócitos isolados, pois tem em conta a influência de outros factores, tais como glóbulos vermelhos, plaquetas e plasma.

Deste modo, linfócitos estimulados com fitohemaglutinina foram expostos a diferentes concentrações de AA e GA e as lesões de DNA quantificadas através do ensaio das SCEs e dos níveis de aductos de DNA (Capítulo 5). A partir destes estudos e

em relação ao ensaio das SCEs, observou-se mais uma vez que a GA é muito mais genotóxica do que a AA, aumentando a GA o nível de SCEs em cerca de 10 vezes, quando comparada com o controlo, o que está de acordo com os resultados previamente obtidos com células V79. Por outro lado, a AA só induziu SCE para a concentração mais elevada (2000 μM). Do mesmo modo, a quantificação de aductos de DNA, nomeadamente N7-GA-Gua e N3-GA-Ade, efectuados em linfócitos estimulados, originaram resultados coerentes com os obtidos em células V79. Os resultados obtidos mostram uma dose-resposta linear entre os níveis de N7-GA-Gua e as concentrações de GA estudadas (até 750 μM), tendo sido este aducto detectado para a menor dose estudada (10 μM). O aducto (N7-GA-Gua) determinado em linfócitos expostos à GA apresentou uma correlação directa entre os níveis de aductos e a frequência de SCEs, tal como anteriormente já se tinha verificado em células V79.

Por outro lado, foi também avaliado o potencial de lesão do DNA da AA e GA em leucócitos humanos, recorrendo-se à técnica do ensaio do cometa alcalino (Capítulo 6). Os resultados mostraram que a GA aumentou significativamente a % de DNA na cauda do cometa, para concentrações superiores a 250 μM, enquanto a AA não provocou lesões de DNA significativas para as concentrações estudadas (até 1000 μM).

Com base nas curvas dose-resposta obtidas para o ensaio das SCEs e do cometa alcalino, a concentração de 250 μM de GA foi escolhida para ser posteriormente utilizada em ensaios com vários indivíduos, tanto no que diz respeito às SCEs (13 indivíduos) como ao ensaio do cometa (25 individuos). Ambos os ensaios mostraram alguma variabilidade inter-individual que poderá estar relacionada com os diferentes polimorfismos envolvidos na destoxificação e nas vias de reparação de DNA.

Deste modo, o presente estudo teve também como objectivo a identificação de possíveis associações entre as lesões de DNA induzidas pela AA e GA quantificadas através dos ensaios das SCEs e do ensaio do cometa, e biomarcadores de susceptibilidade, considerando os polimorfismos genéticos individuais envolvidos nas vias de destoxificação (*GSTM1*, *GSTT1*, *GSTP1*, *GSTA2* e *EPHX1),* e nas vias de reparação de DNA (Capítulo 5 e 6). No que diz respeito à reparação de DNA, foram estudados diferentes genes envolvidos na via de reparação por excisão de bases (BER), tais como *APEX1*, *MUTYH, OGG1, PARP1, PARP4, e XRCC1*, na reparação por excisão de nucleótidos (NER), tais como *ERCC1*, *ERCC2*, *ERCC4*, *ERCC5*, *ERCC6*,

*RAD23B* e *XPC*, na via de reparação por recombinação homóloga (HRR), tais como *NBS*, *RAD51*, *XRCC2* e *XRCC3,* e na via de reparação por recombinação não homóloga (NHEJ), tais como *Ku80, Lig4* e *XRCC4*.

O nível de lesão de DNA determinado pela frequência de SCEs induzidas pela GA, aponta para uma modulação pelos polimorfismos do *GSTP1* (Ile105Val) e do *GSTA2* (Glu210Ala), mas não por outros polimorfismos associados com as vias de reparação de DNA. Por outro lado, os resultados obtidos com base no ensaio do cometa alcalino sugerem associações entre as lesões de DNA e polimorfismos da via BER (*MUTYH* Gln335His e *XRCC1* Gln39Arg) e da via NER (*XPC* Ala499val e Lys939Gln), considerando os genes isoladamente ou combinados. No entanto, mais estudos devem ser efetuados contemplando uma maior amostragem de indivíduos saudáveis de modo a consolidar os resultados obtidos e compreender os mecanismos envolvidos nas diferenças individuais de resposta a genotóxicos.

Os resultados globais mostraram que a GA é de facto mais genotóxica do que a AA, tendo como referência todos os ensaios efectuados bem como os vários tipos de células estudadas. Adicionalmente, todos os resultados apontam fortemente para que a GA, o metabolito epóxido da AA, seja o principal responsável pelos efeitos genotóxicos da AA. É de salientar também, que apesar dos vários tipos de ensaios utilizados neste trabalho terem demonstrado ser adequados para a compreensão dos mecanismos envolvidos na exposição à AA e GA, a quantificação de aductos de DNA por exposição à GA, nomeadamente N7-GA-Gua, mostrou ser aquele que revela maior robustez em termos de avaliação toxicológica devido à sua elevada sensibilidade. Assim sendo, este biomarcador de dose-efectiva pode e deve ser usado em estudos epidemiológicos em complementaridade com os questionários de frequência alimentar. Adicionalmente este trabalho permitiu tirar conclusões acerca da variabilidade da susceptibilidade individual relativa à destoxificação e reparação das lesões de DNA provocadas pela exposição a estes xenobióticos alimentares.